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(54) Title: PAPILLOMA VIRUS VACCINE

(57) Abstract

A method of providing papilloma virus like particles which may be used for diagnostic purposes or for incorporation in a vaccine for use in relation to infections caused by papilloma virus. The method includes an initial step of constructing one or more recombinant DNA molecules which each encode papilloma virus L1 protein or a combination of papilloma virus L1 protein and papilloma virus L2 protein followed by a further step of transfecting a suitable host cell with one or more of the recombinant DNA molecules so that virus like particles (VLPs) are produced within the cell after expression of the L1 or combination of L1 and L2 proteins. The VLPs are also claimed per se as well as vaccines incorporating the VLPs.

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TITLE

PAPILLOMA VIRUS VACCINE

FIELD OF INVENTION

THIS INVENTION relates to papilloma viruses and in particular antigens and vaccines that may be effective in treatment of infections caused by such viruses.

BACKGROUND OF THE INVENTION

Papilloma virus infections are known not only in humans but also in animals such as sheep, dogs, cattle, coyotes, wolves, possums, deer, antelope, beaver, turtles, bears, lizards, monkeys, chimpanzees, giraffes, impala, elephants, whales, cats, pigs, gerbils, elks, yaks, dolphins, parrots, goats, rhinoceros, camels, lemmings, chamois, skunks, Tasmanian devils, badgers, lemurs, caribou, armadillo, newts and snakes (see for example "Papilloma Virus Infections in Animals" by J P Sundberg which is described in Papilloma viruses and Human Disease, edited by K Syrjanen, L Gissman and L G Koss, Springer Verlag 1987).

It is also known (eg. In Papilloma viruses and Human Cancer edited by H Pfister and published by CRC Press Inc 1990) that papilloma viruses are included in several distinct groups such as human papilloma viruses (HPV) which are differentiated into types 1-56 depending upon DNA sequence homology. A clinicopathological grouping of HPV and the malignant potential of the lesions with which they are most frequently associated may be separated as follows.

In a first group may be listed types 1 and 4 which cause benign plantar warts, types 2, 26, 28 and 29 which cause benign common warts, Types 3, 10 and 27 which cause benign flat warts and Type 7 which causes butcher's warts. This first group of infections occur in normal or immunocompetent individuals.

In a s cond group which ref r to immunocompromis d individuals th re may be list d Typ s 5

and 8 which caus highly malignant macular l sions, Types 9, 12, 14, 15, 17, 19-25, 36 and 46-50 which cause macular or flat lesions which are benign or rarely malignant. These macular lesions are otherwise known as epidermodyplasia verruci formis (EV).

In a third group which infect particularly the genital tract there may be listed Types 6, 11, 34 and 39, 41-44, and 51-55 which cause condylomata which are rarely malignant, Types 13 and 32 which cause benign focal epithelial hyperplasia, Types 16 and 18 which cause epithelial dysplasia and other lesions considerable potential including bowenoid papulosis, and Types 30, 31, 33, 35, 45 and 56 which cause condylomata with intermediate malignant potential. The condylomata appear mostly in the anogenital tract and in particular the cervix. Types 16 and 18 are associated with the majority of <u>in situ</u> and invasive carcinomas of the cervix, vagina, vulva and anal canal. The condylomata may also occur in the aerodigestive tract.

In particular HPV16 is associated with premalignant and malignant diseases of the genito-urinary tract, and in particular with carcinoma of the cervix (Durst et al., PNAS <u>80</u> 3812-3815, 1983; Gissmann et al., J. Invest. Dermatol <u>83</u> 265-285, 1984). Presently, there is no information on the role of humoral responses in the neutralization of HPV16.

The detection of antibodies against HPV16 fusion proteins (Jenison et al., J Virol <u>65</u> 1208-1218, 1990; Köchel et al, Int. J Cancer <u>48</u> 682-688, 1991) and synthetic HPV16L1 peptides (Dillner et al. Int. J Cancer <u>45</u> 529-535, 1990) in the serum of patients with HPV16 infection confirms that there are B epitopes within the capsid proteins of HPV, though few patients have HPV 16L1-specific antibodies identified by these techniques. There is no system for HPV16 propagation in vitro, and human genital l sions produc few HPV16 virions; therefore HPV16 particles hav not been available for

immunological studies.

The animal papilloma virus s may also include bovine papilloma virus (BPV) and in particular types BPV1, BPV2, BPV3, BPV4, BPV5 and BPV6 which are also differentiated by DNA sequence homology. In general the other animal papilloma viruses infect deer, horses, rabbits, dogs, rodents and birds. Papilloma viruses are small DNA viruses encoding for up to eight early and two late genes. (for review see Lancaster and Jenson 1987 Cancer Metast. Rev. p6653-6664; and Pfister 1987 Adv. Cancer Res 48, 113-147). The organisation of the late genes is simpler than the early genes. The late genes L1 and L2 slightly overlap each other in most cases. The putative L2 proteins are highly conserved among different papilloma viruses particularly the sequence of 10 basic amino acids at the C-terminal end. The broad domain in the middle reveals only small clustered similarities. The L1 ORF however appears monotonously conserved in all known cases. (See Syrjanen et al above). The amino acid sequence homology reaches 50% with the comparison between HPV1a, HPV6b, BPV1 and CRPV (Cotton tail rabbit papilloma virus).

In regard to immunotherapy concerning papilloma virus infections prior methods of treatment of warts and epithelial skin lesions have involved the use of surgery which can be painful and traumatic with scarring often a result with the risk that reinfection can occur. Treatment with chemicals has also been used. A common treatment agent is salicylic acid which is the main ingredient in strengths ranging from 10% to 40% in tinctures and plasters. Formalin in strengths of 3% - 20% has also been proposed. Cryotherapy has been used for treatment of skin warts. Gluteraldehyde as a treatment agent has also been used. Podophyllin has also been used with varying success for both skin warts and anogenital condylomata. Th types of surg ry that has been used on anog nital condylomata has includ d surgical

excision, cryosurgery and las r surgery. Th us of interferons has also been proposed (see Syrjanen et al above).

Antibodies to the L1 protein of bovine papillomavirus (BPV) have virus-neutralization activity (Pilacinski et a., 1986) and HPV11 virions can be inactivated in an in vitro model by specific antisera (Christensen and Kreider, J. Virol 64 3151-3156, 1990). There is also some evidence that spontaneous regression of HPV1-induced cutaneous warts is associated with increased humoral immune responses to wart protein (Kirchner, Prog. Med. Virol 33 1-41, 1986).

Vaccines have also been proposed with indifferent success. It has been proposed to use vaccines containing autogenous tumor homogenates [Abcarian et al J. Surg Res 22: 231-236 (1977) Dis Colon Rectum 25:648-51 (1982) Dis Colon Rectum 19: 237-244 (1976)]. However it has recently been advocated that patients should no longer be treated with autogenous vaccines because of the potential oncogenic effect of the viral DNA (Bunney 1986 Br Med J 293 1045-1047).

In relation to production of genetically engineered vaccines this matter has been discussed in Pfister (1990) above and it seems that difficulty has been experienced in obtaining an effective vaccine because of the plethora of different papilloma virus types. Pfister however points out that attention should be directed to the so called early proteins (ie. E1, E2, E3, E4, E5, E6, E7 or E8) because these proteins are most likely synthesised in the proliferating basal cells of a wart infection in contrast to the structural proteins which are expressed in the upper epidermal layers. Therefore according to Pfister (1990) virus capsid protein appears to be limited in relation to use in a vaccine. The use of recombinant vaccinia viruses in *in vitro* test systems for papilloma virus arly prot ins in eukaryotic cells has b en discussed also in Pfister

(1990). This may tak the form of a live vaccine consisting of g netically modified vaccina virus expressing papilloma virus prot ins or n th surface of paraformaldehyde fixed autologous cells infected in vitro with vaccinia recombinants or transfected with other expression vectors. Another strategy for vaccine development as discussed in Pfister (1990) is to use an immune stimulating complex of the glycoside Quil A.

Data on successful proplylactic vaccination exist only for bovine fibropapillomas homogenised homogenate of bovine fibropapillomas and has been shown to provide limited immunity (Olson et al J Am Vet Med Assoc 135, 499 (1959) Cancer Res 22 463 (1962)). A vaccine including an engineered L1 fusion protein (Pilacinski et al. UCLA Symp. Molecular and Cellular Biology New Series Vol 32 papilloma Viruses Molecular and Clinical Aspects Alan R Liss New York 1985 257) has also been used in calves but proved unsuccessful in humans (Barthold et al J. Am Vet Med Assoc. 165, 276, 1974). In Pfister (1990) it is stated that there is presently no evidence for a possible prevention of HPV infection by the use of a capsid protein vaccine, but induction of an antitumor cell immunity appears to be feasible.

The L1 and L2 genes have been the basis of vaccines for the prevention and treatment of papilloma virus infections and immunogens used in the diagnosis and detection of papilloma viruses (International Patent Specifications WO8605816 and WO8303623). However, it appears that no commercial usage of these vaccines have taken place.

SUMMARY OF THE INVENTION

Therefore it is an object of the invention to provide virus like particles (VLPs) which may be useful as diagnostic agents as well as forming a component of a vaccine for use with papilloma virus infections.

Th invention therefore in one asp ct includes a method for production of papilloma virus like particl s

(VLPs) including the st ps f:

(i) constructing one or more recombinant DNA molecules which each ncode papilloma virus L1 prot in or a combination of papilloma virus L1 protein and papilloma virus L2 protein; and

(ii) transfecting a suitable host cell with said one or more recombinant DNA molecules so that virus like particles (VLPs) are produced within the cell after expression of the L1 or combination of L1 and L2 proteins.

The invention in another aspect includes a vaccine containing the papilloma virus VLPs in combination with a suitable adjuvant.

In relation to step (i) only papilloma virus L1 protein is required to form VLPs of some papilloma viruses. Suitably only the L1 protein is required to form VLPs of BVP1, HPV11 and HPV6 including HPV6b. However VLPs may also be formed in relation to BPV1, HPV11 or HPV6b containing both L1 and L2 proteins. For the formation of VLPs of other papilloma viruses such as HPV16, both the L1 and L2 proteins are required. This situation is also believed applicable to HPV18 which has similar pathological symptoms to HPV16 and also similar DNA sequence homology. Further it will be appreciated that the L1 and L2 genes may be included in the same DNA recombinant molecule or in different DNA recombinant molecules.

Preferably the recombinant DNA molecules are contained in recombinant virus which may transfect the host cell. Suitable viruses that may be used for this purpose include baculovirus, vaccinia, sindbis virus, SV40, Sendai virus, adenovirus, retrovirus or poxviruses. Suitable host cells may include host cells that are compatible with the above viruses and these include insect cells such as Spodoptera frugiperda, CHO cells, chicken embryo fibroblasts, BHK cells, human SW13 cells, drosophila, mosquito cells deriv d from Aedes albopictus

or monkey epithelial cells. It will also be appr ciated that oth r ukaryote c lls may comprise y ast cells or other mammalian c lls.

The DNA recombinant molecule is suitable obtained from a source of papilloma virus genome whereby L1 protein or L2 protein may be amplified by PCR amplification using suitably designed primers discussed hereinafter. Preferably a gene encoding L1 protein is inserted in a plasmid containing a suitable promoter and a DNA fragment containing the L1 protein and promoter is incorporated in a primary plasmid which may constitute the recombinant DNA molecule which may be inserted into a recombinant virus vector as described above.

A gene encoding the L2 protein may also be linked to a suitable promoter and preferably a DNA fragment incorporating the L2 gene and promoter is inserted into the primary plasmid to provide a doubly recombinant plasmid or secondary plasmid which plasmid may also be inserted in a recombinant virus vector as described above to form a doubly recombinant virus vector.

However the invention also includes the embodiment wherein the primary plasmid and/or the secondary plasmid may infect a suitable host cell to produce VLPs containing L1 protein or VLPs containing L1 and L2 protein under appropriate experimental conditions. The latter VLPs are the ideal immunogen for a papilloma virus specific vaccine, as the L2 protein is immunodominant in natural infection.

Other suitable DNA recombinant molecules include cosmids as well as recombinant viruses. Suitable expression systems include prokaryotic expression systems including <u>E coli</u> and any plasmid or cosmid expression vector or eukaryotic systems including host cells described above in combination with a recombinant virus vector or alternatively yeast cells and yeast plasmids.

In the situation where plasmids are used which

incorporate genes ncoding L1 or both L1 and L2 and wherein such plasmids may inf ct a suitable host cell for production of VLPs such plasmids should also include a suitable promoter to enhance expression of the VLP structural proteins and a polymerase may also be utilised which is associated with the relevant promoter. However in this situation VLPs may only be obtained under specific experimental conditions.

The L1 and L2 genes may be driven off any mammalian or viral promoter with a mammalian or viral polyadenylation signal. Preferably the L1 and L2 genes are transcribed from any vaccinia virus promoter which may be an early promoter or late promoter as considered appropriate. A list of such promoters is given in Davision and Moss (1989) J. Mol. Biol <u>210</u> 749-769 and (1989) J. Mol. Biol <u>210</u> 771-784.

In the experimental work that has taken place the L1 gene is located downstream of a vaccinia 4b promoter and the L2 gene is located downstream of a synthetic vaccinia 28k late promoter. The host cell is monkey epithelial cells.

The VLPs may be obtained from the transfected cells by any suitable means of purification. The VLPs may be combined with any suitable adjuvant such as ISCOMS, alum, Freunds Incomplete or Complete Adjuvant, Quil A and other saponins or any other adjuvant as described for example in Vanselow (1987) S. Vet. Bull. <u>57</u> 881-896.

Reference may now be made to various preferred embodiments of the invention as illustrated in the attached drawings. In these preferred embodiments it should be noted that the specific papilloma viruses, VLPs and specific constructs of DNA recombinant molecules are given by way of example.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a sch matic r pr s ntation of plasmids used to construct HPV16 recombinant vaccinia

viruses.

Figure 2A is a w st rn blot analysis of recombinant HPV16 L1 in vaccinia virus infected CV-1 cells.

Figure 3 is a northern blot analysis of recombinant vaccinia virus infected CV-1 cells.

Figures 4A and 4B are electron microscopy of HPV virus-like particles from CV-1 cells infected with recombinant vaccinia virus.

Figure 5 illustrates CsCl equilibrium density-gradient sedimentation of HPV16 empty capsid.

Figure 6 illustrates Glycosylation of L1 proteins in purified virus particles.

Figure 7 is a flow diagram of the constructions of plasmid pLC200 encoding L1 and pLC201 encoding L1 and L2.

Figure 8 is a western blot analysis of the reactivity of murine sera with baculovirus recombinant L1 protein.

Figures 9 and 10 illustrate mapping results for sera from BLAB/c, C57B1/6, and CBA mice immunized with synthetic HPV 16 capsids and pooled CFA immunised control sera.

Figures 11 and 11A illustrate reactivity of two MAbs specific for L1 with the series of overlapping peptides of the HPV16 L1 molecule.

Figure 11B illustrates antigenic index prediction of HPV16 L1.

Figure 12 shows an epitope map of HPV16 L1.

Figure 13A illustrates synthetic HPV16 VLPs as used for immunisation.

Figure 13B shows reactivity of the purified VLPs with anti HPV16 L2 antiserum by western blot.

Figure 13C illustrates plasmids used to construct recombinant vaccinia viruses in relation to BPV1.

Figures 14A and 14B show analysis of BPV1 L1

and L2 expression in CV-1 cells infected with wild type and recombinant vaccinia viruses; and

Figures 15A and 15B shows electron microscopy of BPV1 capsids obtained from cells infected with recombinant vaccinia virus.

EXAMPLE 1: VLPS DERIVED FROM HPV16

The HPV-16 L1 gene, from the second ATG (nt5637), was amplified by polymerase chain reaction from pHPV16 (provided by Dr. L. Gissmann), using following primers:

1/ 5'-CAGATCTATGTCTCTTTGGCTGCCTAGTGAGGCC-3'

2/ 5'-CAGATCTAATCAGCTTACGTTTTTTGCGTTTAGC-3'

The first methionine codon and stop codon are indicated by underline, and BglII sites were included to facilitate subcloning. The amplified 1527 bp fragment was extracted with phenol and purified by 1% agarose gel electrophoresis. After digestion with BglII the L1 gene was subcloned into the BamHI site of the RK19 plasmid (Kent 1988 Ph.D. thesis, University of Cambridge) which contains a strong vaccinia virus promoter (4b). The resulting plasmid was sequenced (Sanger et al, 1977, Proc. Natl. Acad. Sci. USA 74,5463-5467) and used to prepare a fragment containing the HPV16 L1 gene linked to the 4b promoter by digestion with MluI and Sstl. This fragment was blunted with T4 DNA polymerase and cloned into the Bam HI site of the vaccinia intermediate vector pLC1, which contains the B24R gene of vaccinia virus (Kotwal and Moss, 1989, J. Virol. 63, 600-606; Smith et al, 1989, J. Gen. Virol. 70, 2333-2343), an *E. coli gpt* gene (Falkner and Moss, 1988, J. Virol. 64, 1849-1854; Boyle and Coupar, 1988, Gene 65, 123 - 128) and multiple cloning sites to produce plasmid pLC200.

The HPV16 L2 gene was prepared by partial digestion of pHPV16 with Accl to produce a fragment (4138nt-5668nt) which was filled with Klenow and linked to synthetic BamHl linkers. This L2 fragment was clon d into the Bam HI site of a pUC derived plasmid term d p480

which has a synthetic vaccinia 28K late promot r, with some modifications (Davison and Moss, 1989, J. Mol. Biol. 210, 771-784). The promot r s quenc is as follows:

5'-GAGCTCTTTTTTTTTTTTTTTTTTTTTGGCATATAAATGGAGGTACCC-3'

the late promoter motif is underlined. A fragment containing the L2 gene linked to the 28K promoter was isolated by digestion with SstI/SalI, blunted by T4 DNA polymerase and then cloned into the SstI and SalI sites of pLC200 to produce pLC201(Fig.1).

In Figure 1, HPV16 L1, L2 (open boxes) are under control of vaccinia late promoters (solid boxes). E. coli gpt gene (shaded box) is used as selection marker. Flanking sequence for homologous recombination. The direction of transcription is indicated by arrows.

The pLC201 plasmid was then used to construct a recombinant vaccinia virus as previously described (Mackett et al, 1984, J. Virol. 49, 857-864). Recombinant virus pLC201VV and pLC202VV were selected by plaque assay in the presence of mycophenolic acid, xanthine, and hypoxanthine (Falkner and Moss, 1988). Recombinant vaccinia virus (VV) expressing HPV16L1, and HPV16L2, were prepared and used as previously described (Zhou et al, 1990, J. Gen. Virol. 71, 2185-2190).

Recombinant plasmid pLC201 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. on 27 March, 1992 and given the designation 75226.

Recombinant vaccinia virus pLC201VV was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. on 3 April, 1992 and assigned the designation VR2371.

PURIFICATION OF VIRUS-LIKE PARTICLES

CV-1 cells infected with recombinant viruses pLC201VV were harvested in 10 mM Tris(pH 9.0) 32 hr after infection and homogenised with a Dounce homogeniser. Homogenates were clarified by centrifugation at 2000g to remov th cell debris and layered onto a 30% (wt/vol)

sucrose cushion. The pell t form d by centrifugation at 110,000g in a SW38 rotor for 90 min was susp nded in 10 mM Tris pH9.0 and layer d onto a 20-60% discontinuous sucrose gradient. After centrifugation at 100,000g for 18 hrs, 10 equal fractions of 0.25 ml were collected. Samples were mixed with 0.6 ml ethanol. The pellet obtained after centrifugation at 4°C and 12000g for 20 minutes was collected for further analysis. To determine the density of the virus-like particles, equilibrium density-gradient sedimentation was accomplished in CsCl (1.30g/ml). After centrifugation at 125,000 xg for 20 hrs, 11 fractions of 0.25ml were collected. The density of each fraction was determined, and each was examined for virus-like particles by transmission electron microscopy.

In Figure 2A cells were infected at 10 pfu/cell with wt VV (lane 1) and pLC201VV (lane 2) and harvested 48h post infection. L1 proteins was detected with the HPV16 L1 specific MAb Camvir 1. The 57kDa L1 protein is indicated by the arrow. Binding of Camvir1 to the 35 kDa protein in all three lanes is non-specific.

In Figure 3 RNAs extracted from cells infected with pLC201VV (lane 2), pLC202VV(lane 3) which also incorporates genes encoding HPV16 proteins E1 and E4 as well as genes encoding HPV16 L1 and L2 or wt VV (lane 4) were resolved on a 1.2% formaldehyde-agarose gel. RNA was transfered to nylon membrane and hybridised with a ^{32}P-labelled L2 probe. Formaldehyde-treated lambda DNA-Hind III cut marker are shown (lane 1).

ELECTRON MICROSCOPY

CV-1 cells infected with recombinant vaccinia virus were fixed in 3% (vol/vol) glutaraldehyde in 0.1M sodium cacodylate buffer and postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in epoxy resin. Thin section were cut and stained with uranyl acetat and lead citrate. Fractions from th sucrose gradient were dried onto EM grids, and n gativ ly

stained with 1% (wt/vol) phosphotungstic acid (pH7.0). Fractions were examined using a JEOL 1200Ex Transmission el ctron microscope.

In Figure 4A there is shown CV-1 cells infected with pLC201VV for 32 hours. In the CV-1 nuclei, particles of approximately 40-nm diameter (arrowed) were frequently found. The bar corresponds to 100 nm.

In Figure 4B there is shown fraction 5 of the sucrose gradient. Papilloma virus-like particles, apparently consisting of regular arrays of capsomeres were observed (arrowed). The bar corresponds to 50nm.

ANALYSIS OF HPV ORF PRODUCTS

HPV16 L1 expression was analysed by immunoprecipitation and immunoblot. For immunoprecipitation, ^{35}S metabolically labelled recombinant VV infected CV-1 cells were lysed in RIPA buffer 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 0.5 μg/ml aprotinin, 10mM Tris-HCl, pH7.4). Immunoblot analysis of partially purified virus-like particles, using the L1 specific MAb Camvir1 (Mclean et al, 1990, J. Clin. Pathol. 43, 488-492) and ^{125}l anti-mouse IgG(Amersham), was performed as previously described using samples solublised in 2x SDS gel loading buffer containing 2-mercaptoethanol. Analysis of HPV16 L2 gene expression is shown in Figure 13B. For analysis of N-glycosylation, partially purified virus-like particles were taken up to 100 μl buffer (0.25 M sodium acetate, pH6.5, 20 mM EDTA and 10 mM 2-mercaptoethanol) and reacted with 0.5 u Endoglycosidase F (Boehringer Mannheim) at 37°C for 18 hrs prior to immunoblotting.

Mycophenolic acid was used to select a vaccinia virus recombinant for the <u>gpt</u> plasmid pLC201 and this was termed pLC201VV. Synthesis of L1 in cells infected with pLC201VV was confirmed by immunoblotting and immunoprecipation. L1 protein was demonstrated as a band on autoradiography of approximately 57kDa. A north rn blot of RNA extracted from CV-1 c lls inf cted with these

recombinant viruses confirmed high lev ls of L2 mRNA transcription in cells infected with either of these viruses (Fig 3). L2 transcription from a synthetic vaccinia virus late promoter gave a heterogeneous Northern blot pattern because VV late RNAs do not use a specific transcription termination signal.

CV-1 cells were infected with pLC201VV and examined for virus-like particles. Electron micrographs of thin sections of cells infected with pLC201VV, but not of control cells infected only with wild-type vaccinia, showed approximately 40nm virus-like particles in cell nuclei. In most cases these particles were linked in chains, and near the nuclear membrane (Fig. 4a). Cells infected with recombinant vaccinia viruses which expressed HPV16 L1 only or L2 only, and produced the corresponding protein (L1) or mRNA (L2), did not contain virus-like particles. Cells simultaneously infected with two different recombinant vaccinia viruses, which expressed HPV16 L1 and HPV16 L2 respectively, also failed to make any HPV virus-like particles; although L1 protein and L2 mRNA could be identified in pools of these double infected cells simultaneous synthesis of both L1 and L2 within individual cells was not demonstrated.

In Figure 5 HPV16 virus-like particles obtained from CV-1 cells infected with pLC201VV were centrifuged over a sucrose cushion and then subjected to CsCl isopynic sedimentation. Virus-like particles (+) were found in fractions 8 and 9. The transmission electron micrograph of a negatively stained particle from fraction 8 is shown in the insert. The density (g/ml) of each gradient fraction is indicated.

In Figure 6 CV-1 cells were infected with pLC201VV for 32 hrs, and virus-like particles were purified on a sucrose gradient. Samples were precipitated with ethanol and treated with endoglycosidase F before analysis by immunoblotting with an anti-HPV16 L1 antibody Camvir I. Lane 1: purifi d

virus-like particl s; Lane 2 and 3: after tr atment with endoglycosidas F ov rnight. The L1 doubl t is indicated by (=), and deglycosylat d L1 is indicated by the arrow. Molecular weight markers are shown on the left.

To confirm that the virus-like particles observed by electron microscopy contained HPV16 L1 protein, cell extracts from pLC201VV infected cells were subjected to a partial purification in a 20%-60% sucrose gradient. Ten fractions were collected and examined for L1 protein. From fractions 3 to 7, L1 could be detected and in fraction 5, the highest level of L1 was found. Each fraction was also examined by EM for virus-like particles: these were observed in fraction 5. A typical papilloma virus negatively-stained with sodium phosphotungstate, has 72 regular close-packed capsomeres (Finch and Klug, 1965, J. Mol. Biol. 13, 1-12; Rowson and Mahy, 1967, Bacteriol. Rev. 31, 110-131) and has a diameter about 50nm. The diameter of the virus-like particles purified from the infected CV-1 cells varied between 35nm and 40nm. These virus-like particles however possessed a similar EM appearance to papilloma viruses, and a regular array of capsomeres could be recognised (Fig 4b). The virus-like particles identified in fraction 5 of the sucrose gradient were therefore presumed to be empty and incorrectly assembled arrays of HPV capsomeres. In CsCl, HPV16 virus-like particles sedimentated at about 1.31 g/ml(Fig 5), and showed a typical empty papilloma virus capsid appearance under transmission electromicroscope (Fig 5, insert).

Camvir-1 identified a protein doublet in western blots of virus-like particles purified from pLC201VV infected CV-1 cells (Fig 6). HPV16 L1 contains four potential N-glycosylation sites (asparagine 157, 242, 367 and 421). To test whether the doublet represented glycosylation variants of the L1 polypeptide, partially purifi d virus-like particl s were subject d to treatment with ndoglycosidase F, prior to SDS-PAGE and

immunoblotting. This r sult d in the replacem nt of the doublet by a single band of slightly lower apparent molecular weight, at the expected molecular weight of about 57 kDa (Fig 6. lane 2,3).

The virus-like particles collected from fractions of the CsCl gradient with a buoyant density of 1.29-1.30 g/ml were used as antigen in an ELISA assay. All antisera from mice immunised with VLPs were positive (Table 2). Control sera from mice immunized with the similar fractions of a density gradient prepared with lysate of CV-1 cells infected with wild type vaccinia were nonreactive with the virus-like particles. Using two different protocols to coat virus-like particles to ELISA plates (Dillner et al., 1991, J Virol 65, 6862-6871), attempts were made to distinguish reactivity with native HPV virus-like particles from reactivity with the partially denatured proteins of disrupted particles. The murine antisera raised against the VLPs were equally reactive with the native (OD 1.00±0.20) and denatured (OD 1.60±0.45) particles. A panel of 6 monoclonal antibodies specific for defined L1 epitopes included only 1 (Camvir 1) that was weakly reactive (OD 0.064) with native VLPs, and it proved more reactive with denatured particles (OD 0.107) than with native particles, suggesting that the reactivity was with denatured L1 potein in the native VLP preparation.

In Figure 8 ^{125}l-labelled anti-mouse lgG was used as the second antibody. The 57-kDa L1 band is indicated by the arrow. Key: sera from individual mice immunized with VLPs: lanes 1-5, BALB/c; lanes 6-10, C57B1/6; lanes 11-15, B10A;sera from individual mice immunized with CFA; lane 16, BALB/c; lane 17, C57B1/6; lane 18, B10A; anti-HPV 16L1 MAb Camvir 1, lane 19. The molecular weights are indicated on the left.

Reactivity of the anti-VLP antisera with the L1 and L2 proteins of HPV16 was confirmed by immunoblot using baculovirus recombinant HPV16 L1 and L2 proteins.

Sera from all mice immuniz d with th virus-like particles, and each of the monoclonal anti-HPV 16 L1 antibodies, recongized a 57-kDa protein (Fig. 8) in the L1 recombinant-baculovirus-infected S. frugiperda cell lysates, and no comparable reactivity was observed with lysates of S. frugiperda cells infected with wild-type baculovirus. The intensity of reactivity with the L1 protein varied from mouse to mouse, but all sera were reactive with prolonged exposure of the immunoblots. Similar results were obtained with the L2 recombinant-baculovirus-infeced cell lysates: murine anti-VLP antisera and a rabbit antiserum to L2 protein both reacted with a single protein in the lysate. Sera from mice immunized with CFA alone failed to react with protein from lysates of L1 or L2 recombinant-baculovirus-infected S. frugiperda.

EXAMPLE 2: DEFINITION OF LINEAR ANTIGENIC REGIONS OF HPV16L1

PROTEIN USING VLPS

In a further series of experiments the linear antigenic regions of the HPV16 L1 capsid protein using synthetic VLPs were determined. In such experiments mice of three haplotypes ($H-2^d$,$H-2^b$, and $H-2^{d/b}$) were immunized with synthetic HPV16 virus-like particles (VLPs), produced using a vaccinia virus doubly recombinant for the L1 and L2 proteins of HPV16. The resultant anti-VLP antisera recognized HPV16 capsids by ELISA assay and baculovirus recombinant HPV16L1 and L2 protein on immunoblot. Overlapping peptides corresponding to the HPV16L1 amino acid sequence were used to define the immunoreactive regions of the L1 protein. The majority of the L1 peptides were reactive with IgG from the mice immunized with the synthetic HPV16 capsids. A computer algorithm predicted seven B epitopes in HPV16 L1, five of which lay within peptides strongly reactive with the murine antisera. Th murine anti-VLP antis ra failed to react with the two peptides r cognized by anti-HPV16L1

monoclonal antibodies rais d by oth rs against recombinant L1 fusion protein. We conclud that th immunoreactive epitopes of HPV16 d fin d using virus-like particles differ significantly from those defined using recombinant HPV16L1 fusion proteins, which implies that such fusion proteins may not be the antigens to look for HPV16L1 specific immune responses in HPV-infected patients.

<u>Production of HPV16 capsids.</u> Plasmid pLC201 containing HPV16L1 and L2 open reading frames (ORFs) under the control of vaccinia virus promoters 4b (natural) and p480 (synthetic) was used to construct the recombinant vaccinia virus (rVV) pLC201VV as previously described but with exceptions as mentioned below. HPV16 virus-like particles were prepared from pLC201VV-infected CV-1 cells as mentioned previously, but cells were cultured in medium containing rifampicin at 100 μg/ml to prevent the assembly and maturation of vaccinia virus (Moss, "Virology" p685-703 Raven, New York, 1985; Karacostas et al., PNAS 86, 8964-8967, 1989). The infected cells were harvested and lysed by freezing and thawing following Dounce homogenization in 10 mM Tris-HCl (pH 9.0). Lysates were clarified by centrifugation at 2000 g and then spun at 100,000 g for 2 hr over a 20% sucrose cushion in PBS buffer. The pellet was mixed with CsCl to an initial density of 1.30 g/ml and centrifuged at 100,000g for 18 hr at 18°. Fractions were collected and immunoblots were performed on ethanol-precipitated proteins. Fractions testing positive for L1 protein were pooled, and the presence of virus-like particles confirmed by electron microscopy as described above.

<u>Production of antisera.</u> Groups of five mice BALB/c (H-2^d), C57Bl/6 (H-2^b), and B10A (H-$2^{b/d}$) were immunized with CsCl gradient-purified HPV16 virus-like particles. Animals were inoculated with 5 μg of capsid protein by subcutaneous inj cti n. Th initial injection was given with Freund's complete adjuvant, and thr

further injections at 3 w ks' int rvals w r given in saline. Fourteen days aft r the fourth inj ction, sera w re collected and stored at -20°. Material prepared from CV-1 cells infected with wild type vaccinia virus, and processed exactly as for pLC201VV infected cells, was used to immunize control groups of mice according to the same protocol.

<u>Peptides</u>. A series of 15-mer peptides, overlapping by five residues, and spanning the deduced amino acid sequence of HPV16L1 protein (Seedorf et al., 1985, Virology <u>145</u> 181-185; Parton, 1990, Nucleic Acids Res <u>18</u> 363) was synthesized with the DuPont RaMPS multiple peptide synthesis system using Fmoc chemistry according to standard protocols (Fields and Noble, 1990 Int. J. Pept. Protein Res <u>35</u> 161-214) and then conjugated with glutaraldehyde to bovine serum albumin (BSA). To denote the position of the amino acids (aas) in the L1 protein, the putative first initiation codon was designated amino acid number 1 (Table 1). For technical reasons the C-terminal peptide corresponding to aas 521-531 was not used. All peptides used were of greater than 85% purity as judged by HPLC analysis.

<u>Recombinant L1 + L2 proteins</u>. Recombinant baculoviruses expressing the HPV16L1 or the HPV16L2 ORF were used to infect insect SF9 cells. After 3 days incubation at 25°, cells were pelleted by centrifugation at 14,000 g for 5 min. The pellet was dissolved in RIPA buffer (20 mM Tris-HCl, pH 7.6; 2 mM EDTA; 50mM NaCl; 1% deoxycholate; 1% Triton X-100; 0.25% SDS; 1% aproptinin; 1 mM PMSF).

<u>Western blotting</u>. Virus like particles or recombinant L1 or L2 protein were mixed with 2X loading buffer containing 2% SDS/DTT and boiled for 5 min. The proteins were separated in 10% polyacrylamide gels and blotted onto nitrocellulose (Towbin et al., 1979, Virology <u>175</u> 1-9). Filt rs were cut into strips, incubated in 3% BSA in PBS at 37° for 1 hr. Blocked

strips were exposed to the various murine antisera (1:200) or monoclonal antibodies overnight at 4°. The reactive proteins were fisualized by autoradiography after reaction with ^{125}l-conjugated anti-mouse lgG (0.2 μCi/ml)(Amersham).

ELISA assay. Polyclonal antisera were tested for reactivity with synthetic HPV16 capsids by an enzyme-linked immunosorbent assay (ELISA) as previously described (Christensen et al., 1990, PNAS 76 4350-4354, Cowsert et al., 1987, JNC1 79 1053-1057). For assays with "native" synthetic HPV16 capsids, 100 ng of protein in PBS (pH 7.5) was attached to each ELISA plate well (Flow Labs) by incubation for 1 hr at 37°. For assays with "denatured" particles, the particles were suspended in carbonate buffer, pH 9.6, and adsorbed on to the plate overlight at 37°. All subsequent incubations were done at room temperature. The plates were washed with PBS, and unattached sites were blocked by incubation for 1 hr in blocking buffer (5% milk powder in PBS, pH 7.5). The murine antisera (1:200), previously absorbed with wild-type VV-infected CV-1 cell extract, were added and incubated for 1 hr, and the plates were washed with PBS. Horseradish peroxidase-conjugated anti-mouse lgG (Sigma) at 1:1000 dilution in blocking buffer was added and incubated for 1 hr, followed by 10 washes with PBS. Substrate buffer (pH 4.6) containing ABTS (Boehringer) and H_2O_2 was added and the OD415 read after 15 min.

Linear B epitope mapping. B epitopes were identified by screening antisera from immunized animals against the set of overlapping HPV16L1 peptides by ELISA. Synthetic peptides coupled to BSA were diluted in 10 mM sodium carbonate buffer (pH 9.3) and adsorbed to ELISA plates overnight at 4°. Blocking of residual binding sites on the plates was carried out using 3% BSA in PBS for 2 hr at 37°. Diluted mouse antisera (1:500) were incubated with coated plates at room temperature for 2 hr. The plates were wash d with PBS containing Tween 20

(0.1%) and incubat d with peroxidase-conjugated anti-mouse lgG (1:1000) (Sigma) or lgA (1:2000)(Sigma) for 2 hr. Plates w r washed and developed with 0.5mg/ml ABTS in substrate buffer (pH 4.6) for 15 min before recording absorbance values at 415 nm. A peptide was considered reactive if the OD 415 value with the test serum was greater than 3 SDs above the mean for the control serum: this gave a cut-of value of 0.260. An OD 415 of five times the mean OD 415 obtained with control sera (0.55) was arbitrarily considered to define a major reactive epitope.

Monoclonal antibodies and antisera. Five monoclonal antibodies (MAb) raised against HPV16L1 fusion protein were used. MAb 5A4, 1D6, 3D1, and 8C4 (Cason et al., 1989, J. Gen Virol 70 2973-2987) were provided by Dr. Phil Shepherd from London, U.K. and MAb Camvir 1 (McLean et al., 1990, J. Clin. Pathol. 43 488-492) was obtained from Dr. C. McLean (Department of Pathology, University of Cambridge). Rabbit antiserum to HPV16 L2-Trp-E fusion protein was provided by Dr. Denise Galloway (University of Washington, Seattle).

Amino acid sequence analysis and the antigenic index prediction. The antigenic index (Al) (Jameson and Wolf, 1988, Comp. Appl., Biosci 4 181-186) is a measure of the probability that a peptide sequence is antigenic. It is calculated by summing several weighted measures of secondary structure. Values for the predicted HPV16L1 sequence were calculated using PLOTSTRUCTURE software.

In Figures 9 and 10, reactivity (OD 415) of the sera in ELISA with a series of overlapping peptides corresponding to the sequence of HPV16 L1 is shown. Peptide numbers corresponding to the HPV16L1 sequence (see Table 1) are indicated.

In Figures 11 and 11A, the numbering system for the amino acids correspond to HPV16L1 from the first putative initiati n codon. Th r gions with AI value over 1.5 are indicated.

In Figure 12, th regions of HPV16L1 within which B epitopes have been shown to lie in a range of mapping systems are shown. Results with sera from mic immunized with the VLPs (Particles) and with IgA and IgG antibodies in sera from humans with cervical cancer (Human IgA and Human IgG) (Dillner et al. 1990 Int. J Cancer 45 529-535) were obtained using overlapping peptides. The murine anti VLP antisera were held to be significantly reactive with a peptide if the OD was greater than 0.55. Results from rabbits immunized with an L1 fusion protein (Rabbit Serum) (Muller et al. 1990 J Gen Virol 71 2709-2717) are plotted: these were determined using a series of partial-length expression clones and the whole length of the sequence within which the epitope(s) lay is shown. Also indicated are the algorithm-predicted B epitopes (Computer) (Jameson and Wolf Comp. Appl. Biosci 4 181-186 1988) and the epitopes recognised by the published anti-HPV16L1 monoclonal antibodies (Monoclonals) (Cason et al. J. Gen Virol. 70 2973-2987 1989; McLean et al. J. Clin Pathol. 43 488-492 1990). The scale shows the position of the epitopes alond the 531 aa L1 protein, and is numbered from the N-terminal methionine (residue 1). For each epitope containing peptide, the exact location with regard to the N-terminal methionine is also given.

A series of 15-mer synthetic peptides of the HPV16 L1 protein, covering the whole length of the protein with five aa overlaps, was used to define the epitopes in L1 recognized by the various immune sera. Each of 16 antisera from the three tested inbred mouse strains. BALB/c(H-2^d), C57B1/6(H-2^b), and B10A (H-$2^{b/d}$), recognized multiple linear peptides of the L1 protein, and essentially the same peptides from HPV16 L1 were recognized by all strains tested (Fig. 10). Five individual sera were tested from each strain. A peptide was designated reactive if the OD with a serum was great r than the mean + 3SD of th ODs of the negative

control sera; this gave a cutoff for reactivity of 0.260. Sera from mic immunized with CFA alone had an OD 415 reactivity of less than 0.260 with all the L1 p ptid s. While some variation was seen in the intensity of the reactivity of each anti-VLP serum from a given strain with each peptide, each of the peptides was reactive (OD 〉 0.260) with either all or none of the anti-VLP sera from each strain of mouse. The isotype of the peptide-specific antibody in the anti-VLP sera was examined using IgG-and IgA-specific anti-mouse immunoglobulin antibodies. The IgG response was as shown (Figs. 9 and 10) and no significant IgA reactivity could be detected to any peptide (OD 〈 0.050). As most peptides were reactive with the anti-VLP sera an arbitrary OD value of five times the mean negative value was used to define major reactive regions of the L1 protein: the major immunoreactive L1 peptides were evenly distributed along the length of the L1 protein, as seven were in the amino-terminal third of the molecule, seven in the middle third, and eight in the carboxy terminal third. In contrast, the monoclonal antibodies specific for HPV16 L1 recongized single major linear epitopes as previously described (Cason et al., above, McLean et al, 1990 above). Four of the five reactive anti-HPV16L1 monoclonal antibodies (5A4, 1D6, 3D1, 8C4) were reactive with peptide 30 (291-305), whereas Camvir 1 recognized peptide 24 (231-245)(Figs. 11 and 11A). Sera from mice immunized with the virus-like particles failed to react with either of these peptides.

An algorithm was used to deduce likely B epitopes of HPV16L1, based on the predicted protein secondary structure. Possible antigenic regions were calculated as an antigenic index (Al) (Jameson and Wolf, 1988, above) on the basis of chain flexibility, high accessibility and high degree of hydrophilicity (Fig. 11). A region with an Al valu ov r 1.5 was r gard d as a predicted B epitope. Sev n such r gions were found

(amino acids 79-84, 105-108, 120-122, 134-135, 267,269, 298-299, 363-367) and five of thes seven regions were within the 22 peptides to which major reactivity was s en with antisera from mice immunized with synthetic HPV16 capsids. The summary of the B epitope specificity of antisera from different sources is shown in Fig. 12.

In further consideration of Examples 1 - 2 it is noted that papilloma viruses generally produce virions in infected keratinocytes which are readily identifiable by electron microscopy (Almeida et al, 1962, J. Invest, Dermatol 38, 337-345) and which in some cases can be purified and shown to be infectious (Rowson and Mahy, 1967, Bacterial. Reo. 31, 110-131). HPV 16 virions are however, not seen in HPV16 infected cervical epithelial tissue although HPV16 L1 and L2 late gene transcription occurs in differentiated genital epithelium (Crum et al, 1988. J. Virol. 62, 84-90) and L1 translation produces immunoreactive L1 protein in these tissues (Stanley et al 1989, Int. J. Cancer 43, 672-676). In this specification we have shown that expression of HPV16 L1 and L2 genes in epithelial cells is both necessary and sufficient to allow assembly of HPV16 virion-like particles and thus the L1 and L2 proteins of HPV 16 are not defective with regard to virion assembly. The expression of HPV16 late genes in tissues appears to be strictly regulated by the epithelial environment (Taichman et al, 1983, J. Invest. Dermatol 1, 137-140). Failure to detect HPV16 virions <u>in vivo</u>, despite transcription of L1 and L2 and translation of L1, suggests that there is either a post transcriptional block to L2 production in cervical epithelium, or an inhibitor of virion assembly. In the HPV16 containing cell line W12, derived from cervical tissue, virus-like particles were observed when the cells underwent terminal differention <u>in vivo</u> in a murine microenvironment (Sterling et al 1990. J. Virol 64, 6305-6307) suggesting that such c lls hav no bl ck to virion assembly, and that insufficient translation of L2 or

other unknown reasons may explain failure to d monstrate HPV16 virions in cervical tissu s.

Our EM studies show that the mpty HPV16 virion has an average size of about 40 nm which is smaller than other papilloma viruses, but has a similar surface structure compared with other papilloma viruses such as rabbit papilloma virus (Finch and Klug, 1965 J. Mol. Biol 13 1-12), or human wart virus (Rowson and Mahy 1967 above). Sedimentation showed an empty capsid density of about 1.31g/ml, the density expected of empty papilloma virus capside compared with about 1.36g/ml for intact HPV1a virions (Doorbar and Gallimore, 1987, J. Virol. 61, 2793-2799).

The L1 protein from HPV has potential glycosylation sites, and purified BPV particles have minor electrophoretic forms of L1 whose mobility is sensitive to endoglycosidase treatment (Larsen et al, 1987, J. Virol 61, 3596-3601). L2 from HPV 1a and HPV 11 has been observed to be a doublet (Rose et al, 1990, J. Gen. Virol, 71, 2725-2729; Doorbar and Gallimore, 1987 above; Jin et al, 1989, J. Gen. Virol. 70, 1133-1140) and this has been attributed to differences in glycosylation. Our data show that the L1 protein in HPV16 capsomeres is also glycosylated, and that two different glycosylation states exist.

In this specification we used synthetic virus-like particles to study immunogenicity of the HPV16 capsid proteins produced in a eukaryotic system. Capsid proteins produced in eukaryotic cells were used since papilloma-virus capsid proteins produced in eukaryotic cells undergo post-translational modification (Browne et al., 1988 J. Gen. Virol. 69 1263-1273; Zhou et al. 1991 Virology 185 625-632) which may be an important determinant of antigen presentation. A recombinant vaccinia expression vector was chosen because no native HPV16 particles are available from clinical lesions, or from viral propagation in cell cultur . We used the

HPV16 VLPs to produce polyclonal anti-VLP antisera in mice, and these sera reacted strongly with the HPV16 capsids by ELISA. We have demonstrated by immunoblotting that the anti-VLP antisera recognized epitopes in denatured L1 (Fig. 2) and L2. Moreover, anti-VLP sera defined 22 major reactive peptides in a series of fifty-one 15-mer peptides of L1. These data indicate that antisera raised against viral particles nevertheless frequently recognize linear determinants. The profile of humoral reactivity with the set of L1 peptides was almost identical across two MHC disparate mouse strains, suggesting that there are sufficient T epitopes in L1 that MHC restriction is not limiting in determining the humoral response to the HPV16 L1 protein in the mouse strains tested here.

The data for B epitope specificity obtained with our murine anti-VLP antisera can be compared (Fig. 12) with a similar study of "immune" serum from women with cervical dysplasia (Dillner et al., 1990 Int. J Cancer 45 529-535). Several peptides were recognized by both immune human sera and the anti-VLP antisera, but the majority of peptides reactive with the murine anti-VLP antisera were not reactive with the immune human sera (Fig. 12). Neither of the regions of L1 (221-235,291-305) recognized by L1-specific monoclonal antibodies (Cason et al., 1989; McLean et al., 1990) were recognized by our murine anti-VLP antisera. As L1 fusion proteins were used to raise these MAbs, and have also been used to screen for antibody to L1 in human serum, the lack of reactivity of human sera with L1 fusion protein (Jenison et al., 1990 J. Infect. Dis 162 60-69; Köchel et al., 1991 Int. J. Cancer 48 682-688) may be explained by the failure of the L1 fusion proteins to display the epitopes of L1 which are presented to the human immune system by native L1 protein.

Screening for antibodies to the L1 protein with peptides can det ct only linear pitop s. In an attempt

to determine wh ther th r activity in the murine sera was directed against both lin ar and conformational d t rminants we carried out ELISA assays with th particles treated in two ways: one said to preserve native particles and the other to produce denatured protein (Dillner et al., 1991 J. Virol. 65 6862-6871). We did not fine any serum or monoclonal antibody reactive exclusively with particles treated in one or other manner, though one MAB (Camvir 1) reacted more strongly with the denatured that the "native" particles. Lack of reactivity of the majority of the MAbs with the denatured particles suggests that they were only partially denatured, as the same antibodies react with denatured protein in a Western blot. Conversely, the reactivity of Camvir 1 with the native particles is not proof that the linear epitope recognized by this antibody is recognizing denatured L1 protein present in some amount in the native particle preparation, and we have no proof that intact VLPs are preserved under our ELISA conditions.

Since most antibodies recognize conformation dependent determinants (Benjamin et al., 1984 Ann. Rev. Immunol. 2, 67-101), which can involve several noncontiguous polypeptide sequences (Amit et al., 1986 Science 233 747-753), antibodies elicited to virions are unlikely to recognise fused or denatured proteins as well as the native protein, as has been shown for HPV1 antisera (Steele and Gallimore, 1990 Virology 174 388-398). Virions of some skin-wart-associated HPV are available in quantities sufficient for serological assays (Almeida and Goffe, 1965 Lancet 2 1205-1207; Kienzler et al., 1983 Br J. Dermatol. 108 665-672; Pfister and Zur Hausen, 1978 Int. J Cancer 21 161-165; Pyrhsonen et al., 1980 Br. J. Dermatol 102 247-254; Pass and Maizel, 1973 J. Invest. Dermatol 60 307-311) for wart parings. The prevalence of antibodies to purified virions in human immune serum varies from 20 (Genner, 1971 Acta. Derm. Venereol (Stockh) 51 365-373) to 88% (Morison, 1975 Br J

D rmatol 93 545-552) depending on the detection system used. However, until recently, virions of the genital HPV types have be n unavailabl for serological study. The nude mice xenograft system (Kreider et al., 1987 J Virol 61 590-593) has allowed production of HPV11 particles for the detection of human antibodies (Bonnez et al., 1991 J. Gen Virol 72 1343-1347). We anticipate that the HPV16 VLPs described here will allow similar studies of seroreactivity to native HPV16 particles to be developed, and the observed lack of reactivity in human serum to HPV15L1 fusion proteins (Jenison et al., 1991 J Virol 65 1208-1218; Köchel et al., 1991 above) may simply parallel the similar observations with HPV1 (Steele and Gallimore, 1990 Virology 174 388-398).

Antibodies to BPV structural proteins have virus-neutralizing activity (Pilacinski et al., 1986 Ciba Found. Symp. 120 136-156) and antisera raised against purified HPV11 virions could also neutralize infectious HPV11 in an athymic mouse xenograft system (Christensen and Kreider, 1990 J Virol 64 3151-3156). Our results indicated that the purified synthetic HPV16 capsids are immunogenic and could be used to produce and evaluate virus-neutralizing antibodies specific for this oncogenic virus. BPV1 L1 protein expressed in *Escherichia coli* and BPV particles have both protected cattle from development of warts (Pilacinski et al., 1986; Jarrett et al., 1990 Vet. Rec. 126 449-452). A similar immune response to HPV16 virus-like particles would be the basis of a potential vaccine to prevent HPV16-associated cervical cancer.

In Figure 13A, after infection with pLC201VV, CV1 cell lysates were subjected to equilibrium gradient sedimentation. Purified virus-like particles were examined by transmission electron microscopy after negative staining with Phosphotungstic acid. [bar = 100 nm].

In Figure 13B, Lan 1, the lysate from CV1

cells infect d with wild typ virus; lane 2, lysate from L1 and L2 expressing virus pLC201VV; lane 3, purifi d virus-like particles. The L2 protein was probed with a rabbit anti HPV16 L2 antibody followed by ^{125}I-protein A. The molecular weights are indicated on the left and L2 bands are arrowed.

Reference may also be made to Figures 13A and 13B which show that HPV16 L1 and L2 double recombinant VV contain L2 protein as demonstrated by western blot, using purified VLPs and a rabbit antiserum raised against VV recombinant L2 protein.

EXAMPLE 3: BPV1 VLPS

It has also been ascertained that bovine papillomavirus(BPV) 1 virions similarly produced in vitro using VV recombinant for the BPV1 capsid proteins can package BPV1 DNA. Complete virions are able to infect a permissible mouse fibroblast cell line, as indicated by transcription of the E1 viral open reading frame, and infection is inhibited by incubation of virions with antibodies to the capsid protein of BPV1. In contrast to the observations for HPV16, virus like particles assemble in cells infected with VV recombinant for the BPV1 L1 capsid protein alone, but L2 protein is required to package BPV1 DNA to produce infectious virions.

With reference to the HPV16 VLPs referred to above, these particles appeared to consist of capsomeres typical of those seen in HPV1 and BPV1 particles purified from clinical lesions (Bakar et al, J.C. Biphys J <u>60</u> 1445-1456-1991, Staquet et al., J. Dermatologica <u>162</u> 213-219, 1981), though the overall morphology of the HPV16 particles was rather different to naturally occurring HPV1 and BPV1 particles. As natural HPV16 virions have not been purified from clinical lesions, it was considered desirable to ascertain whether this morphological difference was a property of HPV16, or the r combinant vaccinia virus(rVV) system used to produce the virions. A seri s of VVs were therefore made, ach

doubly recombinant for the L1 and L2 capsid proteins of HPV6, HPV11, and of BPV1. Inf ction of CV-1 cells with each of these double recombinant VVs produced virus like particles, and these resembled the authentic HPV1 and BPV1 virions more closely than the HPV16 particles. We chose to study the BPV-1 particles, as natural BPV-1 particles are better characterised morphologically and immunologically (Chen et al, Baker et al 1991, Cowsert et al., J. Natl Cancer Inst. 79 1053-1057) and cell lines are available which are permissive for the episomal replication of BPV-1 DNA (Law et al 1981 DNAS 78 2727-2731).

In Figure 13C, BPV1L1 is expressed from the p4b natural vaccinia late promoter and L2 from the p480 synthetic vaccinia late promoter. The E.coli gpt gene is used as the selection marker. Flanking sequences are the vaccinia B24R gene, which provides a vaccinia sequence for homologous recombination. The BPV1 L1 and L2 genes were cloned by PCR from plasmid pml-1. Because the BPV1 genome is linearised and cloned into this plasmid at a BamHI site in the BPV1 L2 ORF, the BPV1 genome was first isolated from pml-1 by BamHI digestion and recircularised, and the circularised BPV-1 DNA was used as the PCR template. Oligonucleotide primers used for L1 amplification were:

5'-CGGGATCCATGGCGTTGTGGCAACAAGGCCAGAAGCTG.

5'-CGGGATCCTTATTTTTTTTTTTTTTTTGCAGGCTTACTGG.

The BamHI site is underlined and the first methionine and stop condons are in bold.

Oligonucleotide primers for L2 amplification were:

5'-GCAGATCTATGAGTGCACGAAAAAGAGTAAAACGTGCCAGTGC.

5'-GCAGATCTTTAGGCATGTTTCCGTTTTTTTCGTTTCC.

The Bgl II sites are underlined and the first methionine and stop condons are in bold. The amplified 1478 bp L1 fragment was cloned into the BamH1 site in plasmid RK19 to produce RK19BPVL1. The L1 g ne and vaccinia 4b promoter were isolated from this plasmid by dig stion

with MluI and SmaI and transf rred into plasmid pSX3 to produce pSXBPVL1. The 1409 bp L2 fragm nt was digested with Bgl II and cloned into the BamH1 site in plasmid p480 to produce p480BPVL2. The synthetic vaccinia late promoter and BPV L2 gene were cloned from this plasmid into the Sma1 site in pSXBPVL1 to produce the doubly recombinant plasmid pSXBPVL1L2. Transfection of pSXBPVL1 or pSXBPVL1L2 DNA into monolayers of CV-1 infected with wild type (wt) VV WR strain resulted in the rVVs pSXBPVL1VV (L1 expressing) and pSXBPVL1L2VV (L1 and L2 expressing). Recombinant vaccinia viruses were purified three times in presence of mycophenolic acid. Following purification, large-scale preparations of the recombinants were made and used throughout these experiments.

In Figure 14A there is shown immunoprecipitation analysis of recombinant BPV1 L1 in vaccinia-infected cells. CV-1 cells were infected at 10 pfu/cell with wt vaccinia virus (Lane 1); pSXBPVL1VV (lane 2); pSXBPVL1L2VV (lane 3) and harvested 48 hrs postinfection. BPV1L1 protein was detected with BPV1 specific rabbit antiserum. The 58 kDa L1 protein is indicated by an arrow.

In Figure 14B there is shown northern blot analysis of BPV1 L2 expression. RNA extracted from CV-1 cells infected with wt VV (lane 1); pSXBPVL1L2VV (lane 2) was probed with the BPV1 L2 gene. The variable length of the L2 homologous transcripts is typical of transcripts expressed from VV promoters. For immunoprecipitation, cells infected with rVVs were lysed with RIPA buffer (0.1% SDS,1% Triton X-100, 1% sodium deoxcholate, 150 mM NaCl, 0.5μg/ml aprotinin, 10 mM Tris, pH7.4). Soluble proteins were immunoprecipitated with rabbit anti BPV1 antibody (DAKO, Glostrup) at 1:1000 dilution. Precipitated proteins were collected with protein-A sepharose, s parated on 10% SDS polyacrylamide g ls and blotted onto nitrocellulose filters. After blocking with

skim milk, the filt rs were xposed to the anti BPV1 serum(1:1000) foll wed by ^{125}I-prot in A (0.1μCi/ml) and visualised by autoradiography. Total RNA was extracted from cells, and purified by centrifugation through CsCl as described previously. Total RNA, 30μg per track, was run on 1.2% formadelhyde-agarose gels, transferred to nylon membranes, and probed with a ^{32}P-labelled BPV1 L1 gene.

In Figure 15A reference is made to pSXBPVL1L2VV. Some particles from CON/BPV cells infected with pSXBPVL1L2VV have electron dense cores(Insert).

In Figure 15B reference is made to pSXBPVL1VV. A contaminating vaccinia virus in (A) is indicated by a "V". The scale bars represent 50 nm. Cells were harvested two days postinfection, washed with PBS, lysed by freeze and thaw three times and sonicated in PBS. Cell debris was removed by low-speed centrifugation, and the supernants were layered on a 20% (w/v) sucrose cushion and centrifuged for 2 hours at 100,000 x g. The pellets were resuspended in PBS and analysed in a JEOL 1200EX electron microscope after negative staining with 1% (w/v) phosphotungstic acid (pH 7.0).

As the ratio of the L1 to L2 proteins in authentic BPV1 particles is approximately 5:1 (Pfister, H. & Fuchs, E. in Papillomaviruses and human disease (eds Syrjanen, K., Gissman, L & Koss, L.G.) Vol. 1-18 (Springer-Verlag, Berlin, 1987), we used a strong natural promoter for the L1 gene and a weak synthetic promoter for the L2 gene for our doubly recombinant VV (Fig 13C), and the resulting ratio of L1 mRNA to L2 mRNA on a northern blot from rVV infected CV-1 cells was approximately 10:1. CV-1 cells infected with this rVV expressed BPV1 L1 protein (Fig 14A) and L2 mRNA (Fig 14B), and large numbers of 50 nm icosahedral virus-like particles of apparently authentic morphology (Fig 15a) could be purified from the inf ct d c lls. Our previous work with HPV16 had shown that both L1 and L2 prot ins

w r requir d for the assembly of HPV16 virus-like particles as described above, which contrasts with the bs rvation that, for parvovirus (Kajigaya et al, 1991 DNAS 4646-4650), bluetongue virus (Loudon et al, 1991 Virology 182, 793-801), and polyoma virus (Salunke et al, 1986, Cell 46, 895-904), virus like particles assemble in cells if the major capsid protein alone is expressed as a recombinant protein. We therefore produced a VV recombinant for BPV1 L1 (Fig 13C) and observed that when CV-1 cells were infected with this VV, virus like particles of similar morphology to those obtained with the L1 and L2 double recombinant VV could be purified (Fig 15b). Similar empty icosahedral virions were obtained after infection of CV-1 cells with VV recombinant for the L1 proteins of HPV6 or HPV11. The lack of morphologically authentic PV virion in cells infected with the HPV16L1 and L2rVV, and the lack of PV virions seen in HPV16 infected tissue by electron microscopy (Schneider (1987) Papillomaviruses and Human Disease Vol 19-39 Springer Verlag Berlin), suggests that HPV16 in contrast to other PVs may be defective with respect to viral capsid formation.

A minority of virus like particles from cells infected with the VPV1 L1/L2 rVV is shown in the Fig 15a insert.

The cloning strategy for HPV11L1/L2 and HPV6bL1/L2 double expressing recombinant vaccinia viruses is described below:

For HPV11L1:

5'CAGATCTCAGATGTGGCGGCCTAGCGACAGCACAGTATATGTGCC

5'CGGGAATTCGTGTAACAGGACACACATAATAATTGTTTATTGCACAAAA

The PCR product was digested by BgIII/EcoRI and cloned into RK19 under control of 4b promoter. The promoter/11L1 sequence was then cloned into pSX3 BamHl site blunted by Klenow. The resultant plasmid was pSX11L1.

For 11L2

5'GCGGATCCATGAAACCTAGGGCACGCAGACGTAAACGTGCG

5'CGCCCGGGCTAGGCCGCCACATCTGTAAAAAATAAGGG

Th Bam/H1/Sma1 dig st d PCR fragment was cloned into p480 under synthetic 28k late promoter. Then the promoter/11L2 fragment was transferred to pSX11L1. The 11L1/L2 double recombinant expressing plasmid was named as pSX11L1/L2.

For HPV6b11:

5'CGCCCGGGTTACCTTTTAGTTTTGGCGCGCTTACGTTTAGG

5'GCGGATCCAGATGTGGCGGCCTAGFCGACAGCACAGTATATG

The PCR product was cut by BamH1/Sma1 and cloned into RK19 under control of 4b promoter. The promoter/6L1 were then cloned into pSX3 to produce pSX6L1.

For HPV8L2:

The HPV6L2 was isolated from 6b genome by Acc1/Xba1 (4422-5903). The fragment was blotted by klenow and inserted into p480. The synthetic 28k promoter plus 6bL2 was cloned into pSX6L1 to form double recombinant plasmid pSX6L1/L2.

Thereafter plasmids pSX11L1 and pSX11L1/L2 infected a host cell (eg CV1 cells or C127 cells) to produce VV p SX11L1 and VV pSX11 L1/L2 which after transfection of a host cell infected with wild type vaccinia virus formed VLPs containing L1 protein (derived from VV pSX11L1) and L1 and L2 protein (derived from VV pSX11L1/L2). In similar manner VV pSX6L1 and VV pSX6L1/L2 after transfection of a host cell produced VLPs containing HPV6b L1 and VLPs containing HPV6b L1 and HPV6b L2.

It also will be appreciated that the invention includes within its scope viruses doubly recombinant for papillomaviruses capsid proteins L1 and L2 as well as recombinant viruses containing papillomavirus capsid protein L1.

It will further be appreciated that the invention includ s within its scop a method of diagnosis

of papilloma virus inf ction by ELISA including the step of detection of VLP particl s containing proteins L1 and L2.

TABLE 1

15 AA ov rlapping peptides from the predicted sequence of the HPV16 L1 protein

NO	SEQUENCE	RANGE	NO	SEQUENCE	RANGE
1	MQVTFIYILVITCYE	(1-15)	28	SLFFYLRREQMFVRH	(271-285)
2	ITCYENDVNVYHIFF	(11-25)	29	MFVRHLFNRAGTVGE	(281-295)
3	YHIFFQMSLWLPSEA	(21-35)	30	GTVGENVPDDLYIKG	(291-305)
4	LPSEATVYLPPVPVS	(31-45)	31	LYIKGSGSTANLASS	(301-315)
5	PVPVSKVVSTDEYVA	(41-55)	32	NLASSNYFPTPSGSM	(311-325)
6	DEYVARTNIYYHAGT	(51-65)	33	PSGSMVTSDAQIFNK	(321-335)
7	YHAGTSRLLAVGHPY	(61-75)	34	QIFNKPYWLQRAQGH	(331-345)
8	VGHPYFPIKKPNNNK	(71-85)	35	RAQGHNNGICWGNQL	(341-355)
9	PNNNKILVPKVSGLQ	(81-95)	36	WGNQLFVTVVDTTRS	(351-365)
10	VSGLQYRVFRIHLPD	(91-105)	37	DTTRSTNMSLCAAIS	(361-375)
11	IHLPDPNKFGFPDTS	(101-115)	38	CAAISTSETTYKNTN	(371-385)
12	FPDTSFYNPDTQRLV	(111-125)	39	YKNTNFKEYLRHGEE	(381-395)
13	TQRLVWACVGVEVGR	(121-135)	40	RHGEEYDLQFIFQLC	(391-405)
14	VEVGRGQPLGVGISG	(131-145)	41	IFQLCKITLTADVMT	(401-415)
15	VGISGHPLLNKLDDT	(141-155)	42	ADVMTYIHSMNSTIL	(411-425)
16	KLDDTENASAYAANA	(151-165)	43	NSTILEDWNFGLQPP	(421-435)
17	YAANAGVDNRECISM	(161-175)	44	GLQPPPGGTLEDTYR	(431-445)
18	ECISMDYKQTQLCLI	(171-185)	45	EDTYRFVTQAIACQK	(441-455)
19	QLCLIGCKPPIGEHW	(181-195)	46	IACQKHTPPAPKEDD	(451-465)
20	IGEHWGKGSPCTNVA	(191-205)	47	PKEDDPLKKYTFWEV	(461-475)
21	CTNVAVNPGDCPPLE	(101-215)	48	TFWEVNLKEKFSADL	(471-485)
22	CPPLELINTVIQDGD	(211-225)	49	FSADLDQFPLGRKFL	(481-495)
23	IQDGDMVHTGFGAMD	(221-235)	50	GRKFLLQAGLKAKPK	(491-505)
24	FGAMDFTTLQANKSE	(231-245)	51	KAKPKFTLGKRKATP	(501-515)
25	ANKSEVPLDICTSIC	(241-255)	52	RKATPTTSSTSTTAK	(511-525)
26	CTSICKYPDYIKMVS	(251-265)	53	STTAKRKKRKL	(521-531)
27	IKMVSEPYGDSLFFY	(261-275)			

The sequence of each L1 peptide is give using the single letter code. The location of each peptide within the HPV16 L1 protein is given, assigning position 1 to the N terminal methionine. The short C terminal peptide (no 53) was not used for these experiments.

Table 2

Immunoreactivity to virus-like particles of sera from mice immunized with synthetic HPV16 capsids.

	Reactivity with virus-like particles		
Mouse strain	Mice immunised with VLPs		Immunised with CFA
	Expt 1 (n=5)	Expt 2(n=5)	n=2
BALB/C	1.06 ± 0.29*	1.71 ± 0.06	0.36 ± 0.04
B10A	1.08 ± 0.06	1.75 ± 0.04	0.24 ± 0.02
C57Bl/6	0.86 ± 0.20	1.70 ± 0.12	0.25 ± 0.03

* Values are OD 415 units, and are given as the mean ± 1 standard deviatio

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A method of production of a papilloma virus like particles (VLPs) including th steps of:

(i) constructing one or more recombinant DNA molecules which each encode papilloma virus L1 protein or a combination of papilloma virus L1 protein and papilloma virus L2 protein; and

(ii) transfecting a suitable host cell with said one or more recombinant DNA molecules so that virus like particles (VLPs) are produced within the cell after expression of the L1 or combination of L1 and L2 proteins.

2. A method as claimed in claim 1 wherein the DNA moecules encoding the L1 and L2 proteins are included in the same DNA molecule.

3. A method as claimed in claim 1 wherein the DNA molecules encoding the L1 and L2 proteins are included in different DNA recombinant molecules.

4. A method as claimed in claim 1 wherein in step (ii) recombinant viruses containing one or more recombinant DNA molecules transfect the host cell.

5. A method as claimed in claim 4 wherein in step (i) a primary plasmid is formed containing said recombinant DNA molecule(s) which subsquently infect a host cell already infected with a wild type virus to produce said recombinant viruses.

6. A method as claimed in claim 5 wherein said primary plasmid contains said recombinant DNA molecule(s) linked to a promoter to enhance expression of the L1 or L1 and L2 proteins in step (ii).

7. A method as claimed in claim 5 wherein prior to formation of said primary plasmid a gene encoding said L1 or L2 protein is inserted into a secondary plasmid containing said promoter before formation of the primary plasmid.

8. A method as claim d in claim 5 wh rein said primary plasmid which contains said rec mbinant DNA

molecule nc ding L1 prot in has ins rt d th r in a further r combinant DNA mol cul encoding L2 prot in to form a double recombinant expressing plasmid wh reafter both the primary plasmid and the double recombinant expressing plasmid infect the host cell infected with the wild type virus.

9. A method as claimed in claim 4 wherein said recombinant virus are derived from baculovirus which infect an insect cell such as Spodoptera frugiperda.

10. A method as claimed in claim 4 wherein said recombinant viruses are derived from vaccinia virus.

11. A method as claimed in claim 10 wherein a gene encoding the L1 protein is (i) incorporated in a plasmid containing a strong vaccine virus promoter 4b; (ii) a DNA fragment containing the gene linked to the 4b promoter is derived from the plasmid and (iii) subsequently the DNA fragment is inserted in a vaccine intermediate vector which contains the B24R gene of vaccine virus and an E. coli gpt gene and multiple cloning sites to produce plasmid pLC 200.

12. A method as claimed in claim 11 wherein a gene encoding the L2 protein was incorporated in a pUC derived plasmid having a synthetic vaccinia 28K late promoter before obtaining a fragment containing the L2 gene linked to the 28K promoter was inserted into plasmid pLC 200 to produce plasmid pLC 201.

13. A method as claimed in claim 12 wherein plasmid pLC 201 was utilized to construct recombinant vaccinia virus pLC 201VV which subsequently infected mammalian cells to produce VLPs containing proteins L1 and L2.

14. A method as claimed in claim 1 wherein a recombinant DNA molecule is constructed containing a gene encoding BPV L1 protein is linked to vaccinia 4b promoter after PCR amplification whereafter said DNA recombinant molecule is inserted into plasmid pSX3 to form plasmid pSXBPVL1 which th reaft r transf cted a h st c ll inf ct d with wild type vaccinia virus to produc rVV

pSXBPVL1 which after subsequent transfection of a host cell produced a VLP containing BPV L1 prot in.

15. A method as claim d in claim 14 wherein a recombinant DNA molecule is constructed containing a gene encoding BPV L2 protein linked to synthetic vaccinia 28K late promoter after PCR amplification which recombinant DNA molecule is plasmid p480 BPVL2 before insertion into plasmid pSXBPVL1 to produce plasmid pSXBPVL1L2 which thereafter transfected a host cell infected with wild type vaccinia virus to produce a VLP containing BPV L1 protein.

16. A method as claimed in claim 1 wherein a recombinant DNA molecule is constructed containing a gene encoding HPV11L1 protein linked to vaccinia 4b promoter after PCR amplification whereafter said DNA recombinant molecule is inserted into plasmid pSX3 to form plasmid pSX11L1 which thereafter infected a host cell to produce HPV11 VLPs containing L1 protein.

17. A method as claimed in claim 1 wherein a recombinant DNA molecule is constructed containing a gene encoding HPV11L2 protein linked to vaccinia 4b promoter after PCR amplification whereafter said recombinant DNA molecule is inserted into plasmid p480 containing vaccinia 28K late promoter and subsequently the DNA fragment containing the gene linked to the promoter is transferred to plasmid pSX11L1 to form plasmid pSX11L1L2 which thereafter infected a host cell to produce VVpSX11L1L2 which after transfection of a host cell produced VLPs containing L1 and L2.

18. A method as claimed in claim 1 wherein a recombinant DNA molecule is constructed containing a gene encoding HPV6bL1 protein linked to vaccinia 4b promoter which is subsequently inserted in plasmid pSX3 to produce plasmid pSX36L1 which thereafter infected a host cell to produc VV pSX36L1 which after transfection of a host cell form d VLPs containing HPV6bL1.

19. A method as claimed in claim 1 wherein a

r combinant DNA m l cul is c nstruct d containing a gen encoding HPV6bL2 prot in linked to vaccinia 28K lat promoter which thereaft r was clon d into pSX36L1 to form double recombinant plasmid pSX6L1L2 which thereafter infected a host cell to produce VV pSX36L1L2 which after transfection of a host cell formed VLPs containing HPV6bL1 protein and HPV6bL2 protein.

20. A method of production of a vaccine including the step of combining the VLPs of any one of claims 1 - 20 with an adjuvant.

21. VLPs produced from the method of any one of claims 1 - 20.

22. A vaccine produced by the method of claim 21.

23. VLPs derived from papilloma virus containing proteins L1 and L2.

24. VLPs derived from HPV16 containing proteins L1 and L2.

25. VLPs derived from BPV1 containing proteins L1 and L2.

26. VLPs derived from BPV1 containing protein L1.

27. VLPs derived from BPV1 containing proteins L1 and L2.

28. VLPs derived from HPV11 containing protein L1.

29. VLPs derived from HPV6b containing proteins L1 and L2.

30. VLPs derived from HPV6b containing protein L1.

31. Plasmid PLC 201.

32. Viruses doubly recombinant for papilloma virus capsid proteins L1 and L2.

33. Viruses recombinant for papillomavirus capsid protein L1.

34. Plasmid PLC 200.

35. 5′ - CAGATCTATGTCTCTTTGGCTGCCTAGTGAGGCC - 3′

36. 5′ - CAGATCTAATCAGCTTACGTTTTTTGCGTTTAGC - 3′

37. PLC 201 VV.

38. Polyclonal anti-VLP HPV16 antisera.

39. B epitopes shown in Fig. 12.

40. VVs doubly recombinant for the L1 and L2 proteins of HPV6 and HPV11.

41. 5' - CGGGATCCATGGCGTTGTGGCAACAAGGCCAGAAGCTG

42. 5' - CGGGATCCTTATTTTTTTTTTTTTTTTTGCAGGCTTACTGG

43. 5' - GCAGATCTATGAGTGCACGAAAAAGAGTAAAACGTGCCAGTGC

44. 5' - GCAGATCTTTAGGCATGTTTCCGTTTTTTTCGTTTCC

45. pSXBPVL1.

46. p480BPVL2.

47. pSXBPVL1L2.

48. pSXBPVLIVV.

49. pSXBPVL1L2VV.

50. pXS11L1.

51. pSX11L1L2.

52. pSX6L1.

53. pSX6L1L2.

54. VV pSX11L1.

55. VV pSX11L1L2.

56. VV pSX6L1.

57. VV pSX6L1L2.

58. 5'CAGATCTCAGATGTGGCGGCCTAGCGACAGCACAGTATATGTGCC

59. 5'CGGAATTCGTGTAACAGGACACACATAATAATTGTTTATTGCACAAAA

60. 5'GCGGATCCATGAAACCTAGGGCACGCAGACGTAAACGTGCG

61. 5'CGCCCGGGCTAGGCCGCCACATCTGTAAAAAATAAGGG

62. 5'CGCCCGGGTTACCTTTTAGTTTTGGCGCGCTTACGTTTAGG

63. 5'GCGGATCCAGATGTGGCGGCCTAGCGACAGCACAGTATATG

64. A method of diagnosis of prior infection of papillomavirus by ELISA including the step of detection of VLP particles containing proteins L1 and L2.

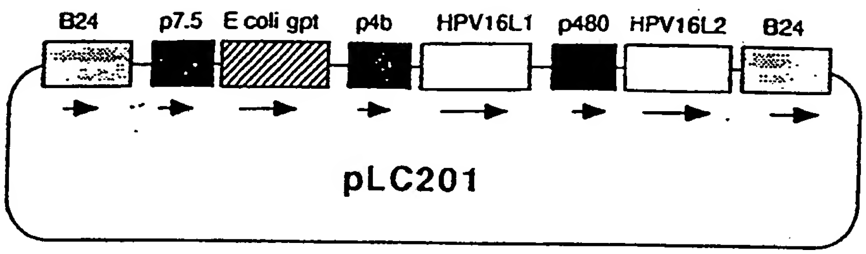


FIG 1

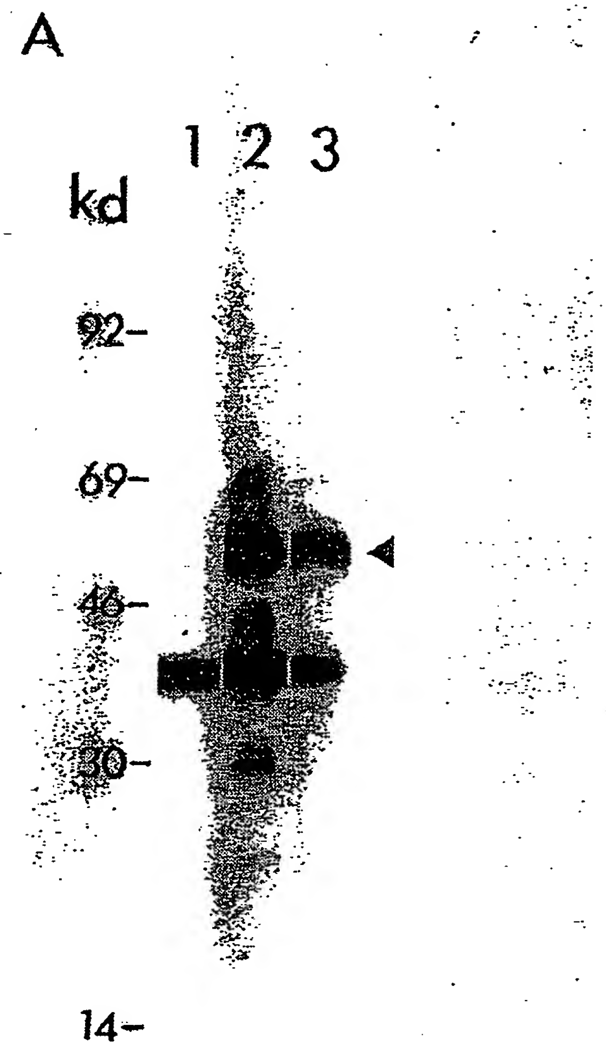


FIG 2A

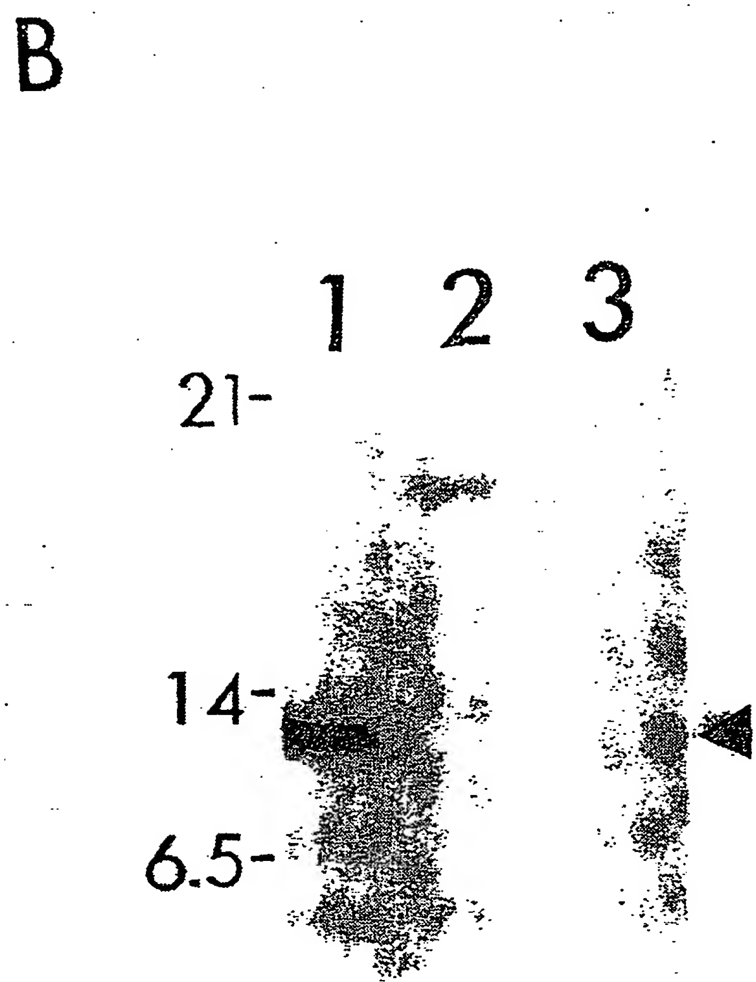


FIG 2B

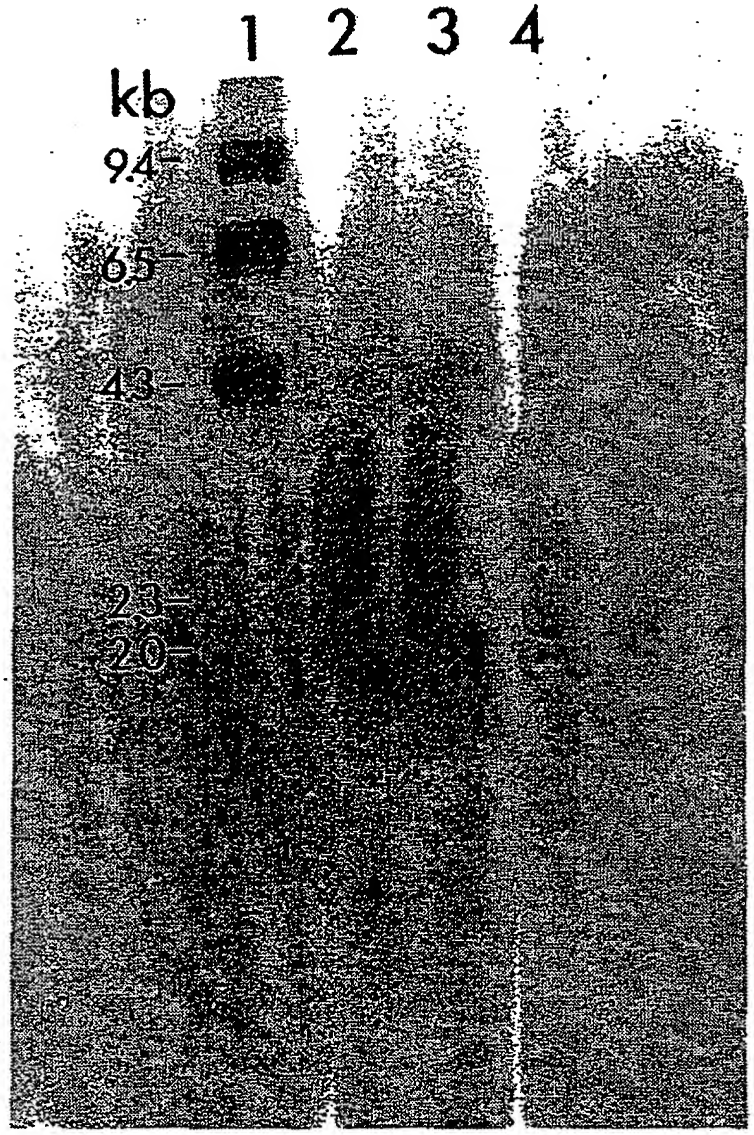


FIG 3

FIG 4A

FIG 4B

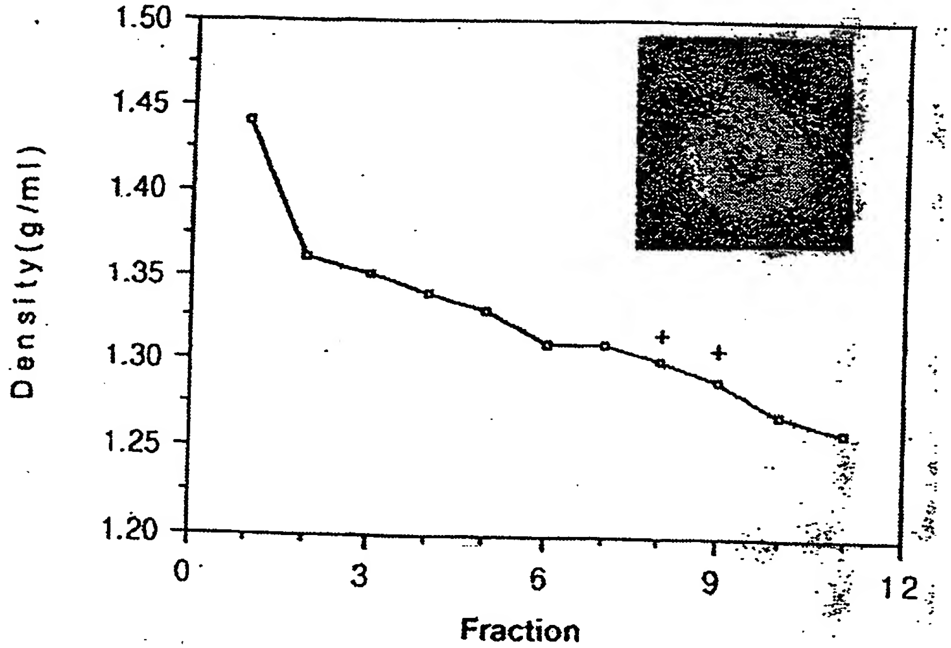


FIG 5

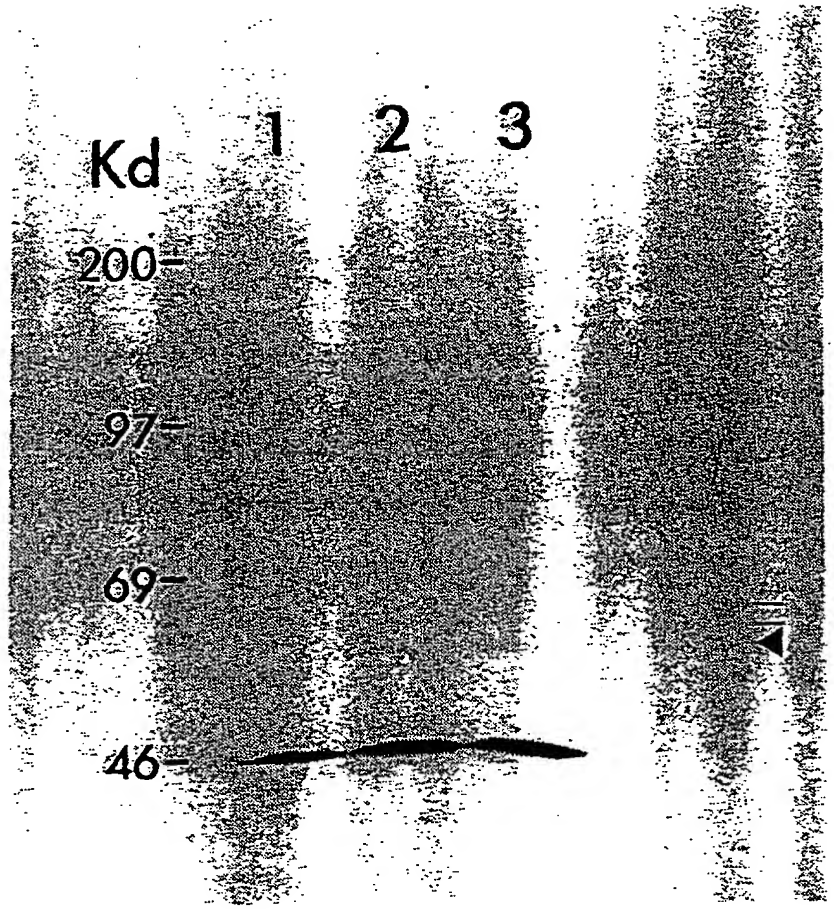
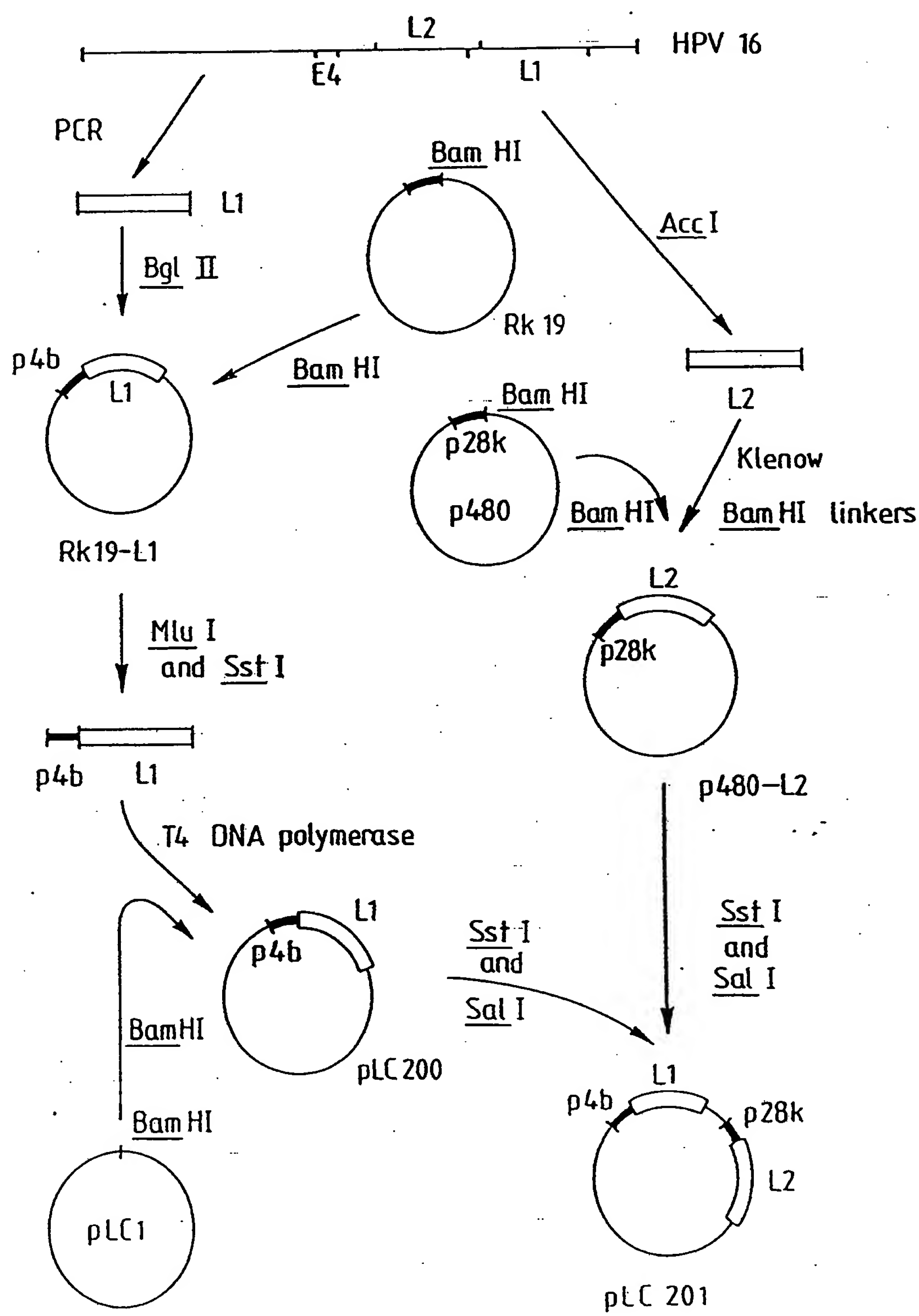


FIG 6

Fig. 7.


L2
HPV 16
E4
L1
PCR
Bam HI
L1
Acc I
Bgl II
Rk 19
p4b
L1
Bam HI
L2
Bam HI
p28k
Klenow
p480
Bam HI
Bam HI linkers
Rk19-L1
L2
p28k
Mlu I
and Sst I
p4b
L1
p480-L2
T4 DNA polymerase
L1
p4b
Sst I
and
Sal I
Sst I
and
Sal I
BamHI
pLC200
L1
p4b
p28k
BamHI
L2
pLC1
pLC 201

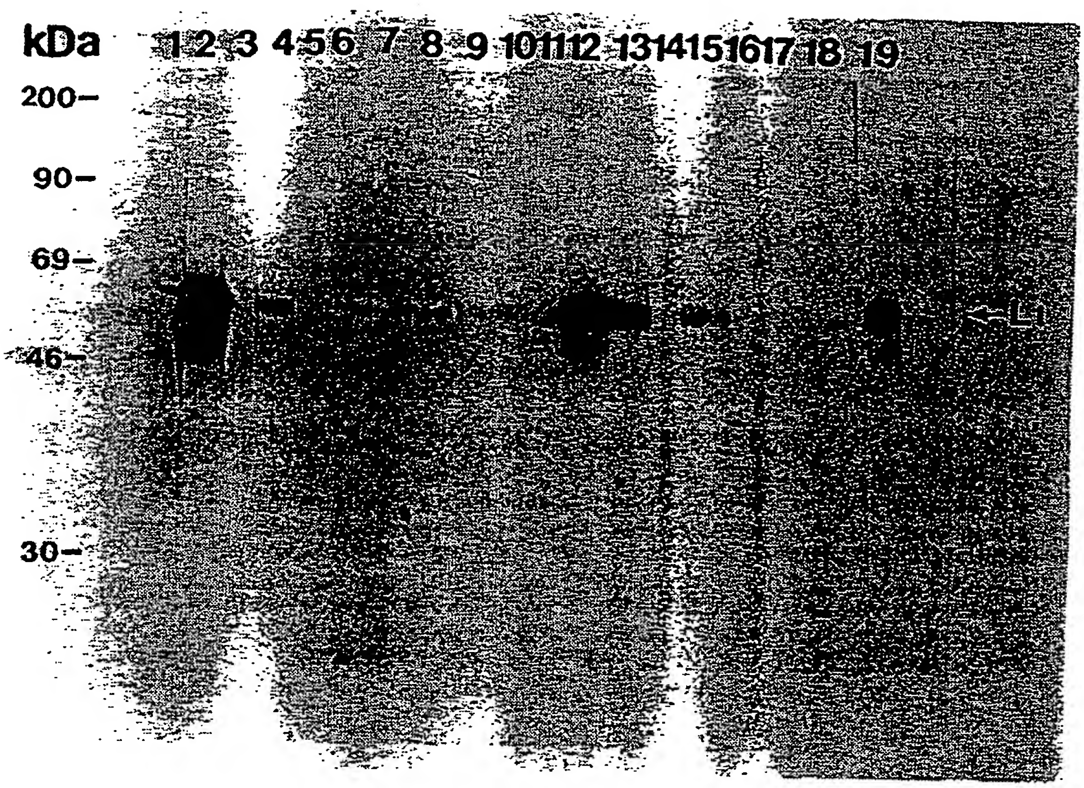


FIG 8

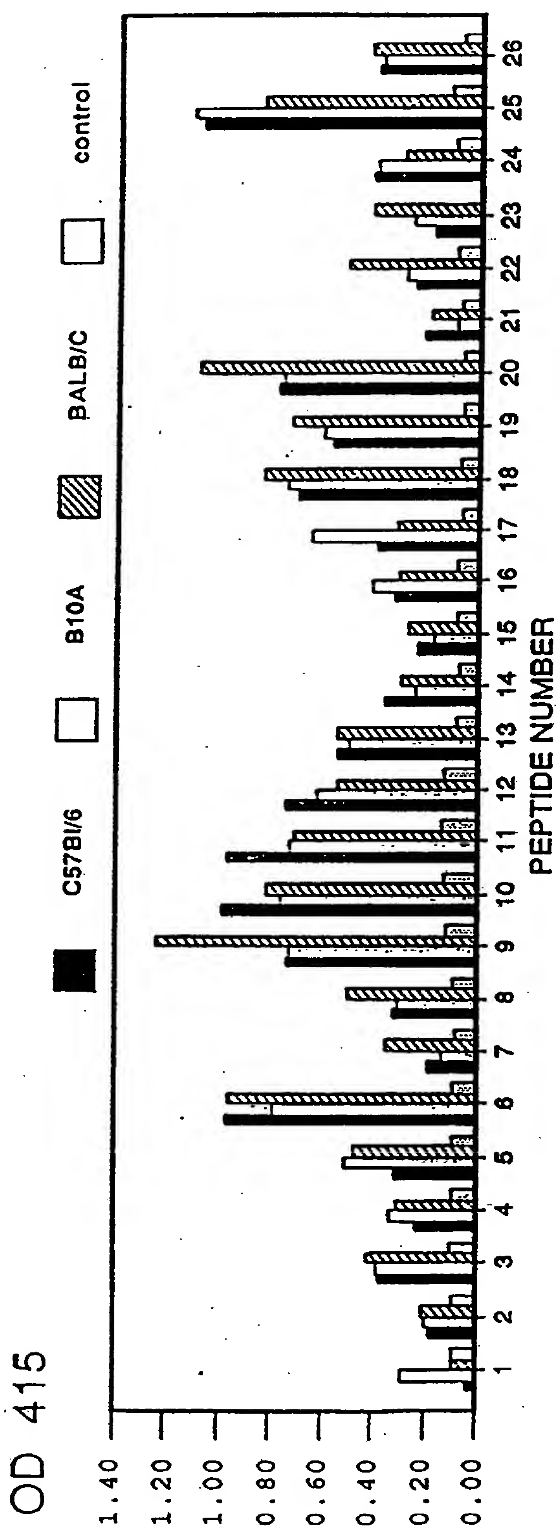


FIGURE 9

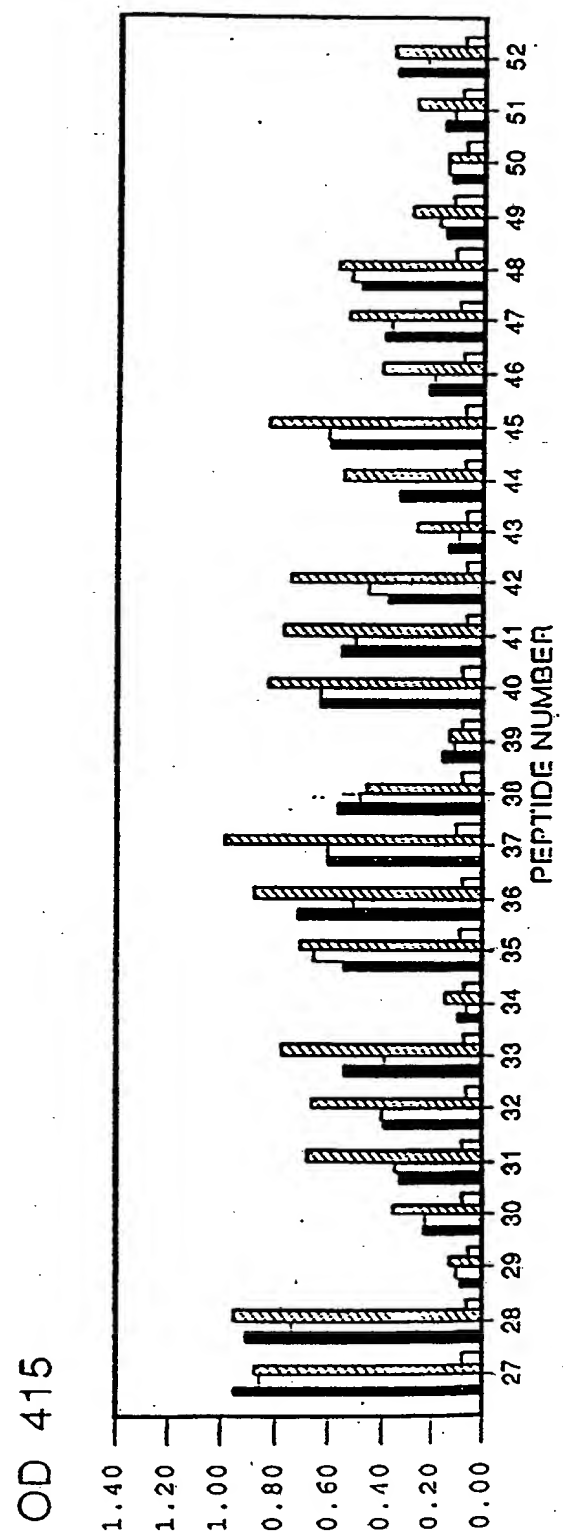


FIGURE 10

Figure 11

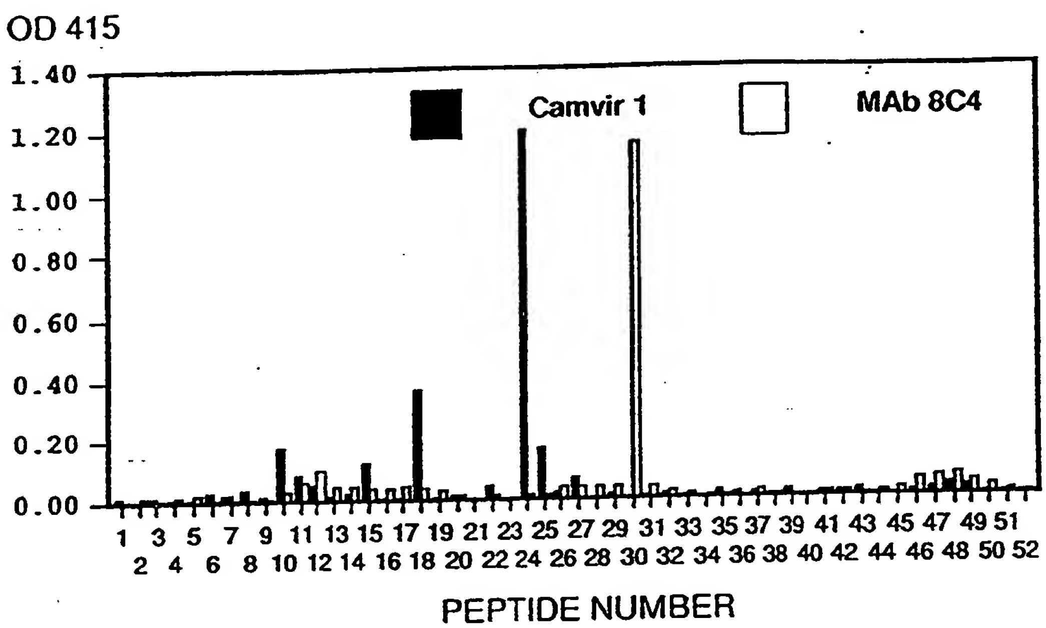
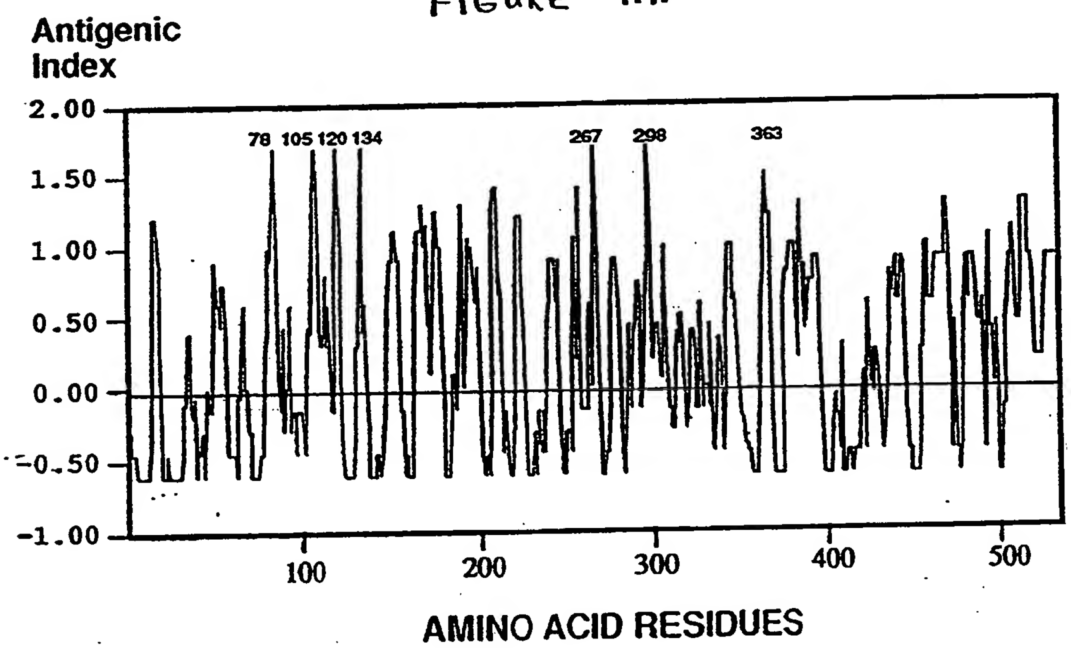


FIGURE 11A



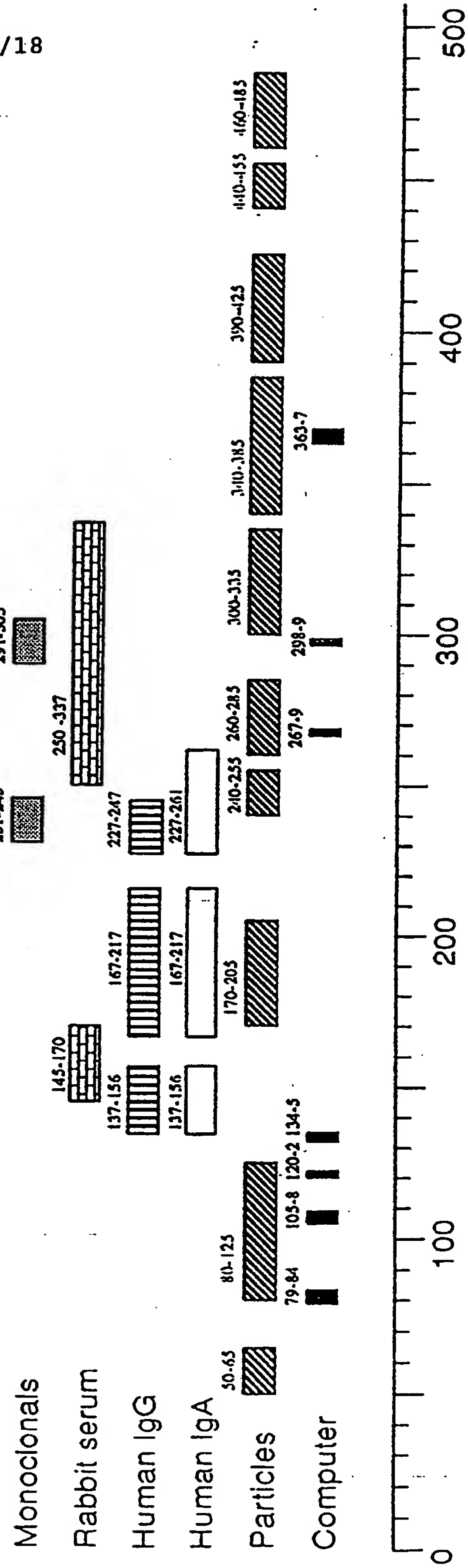


FIGURE 12

SUBSTITUTE SHEET

FIG 13A

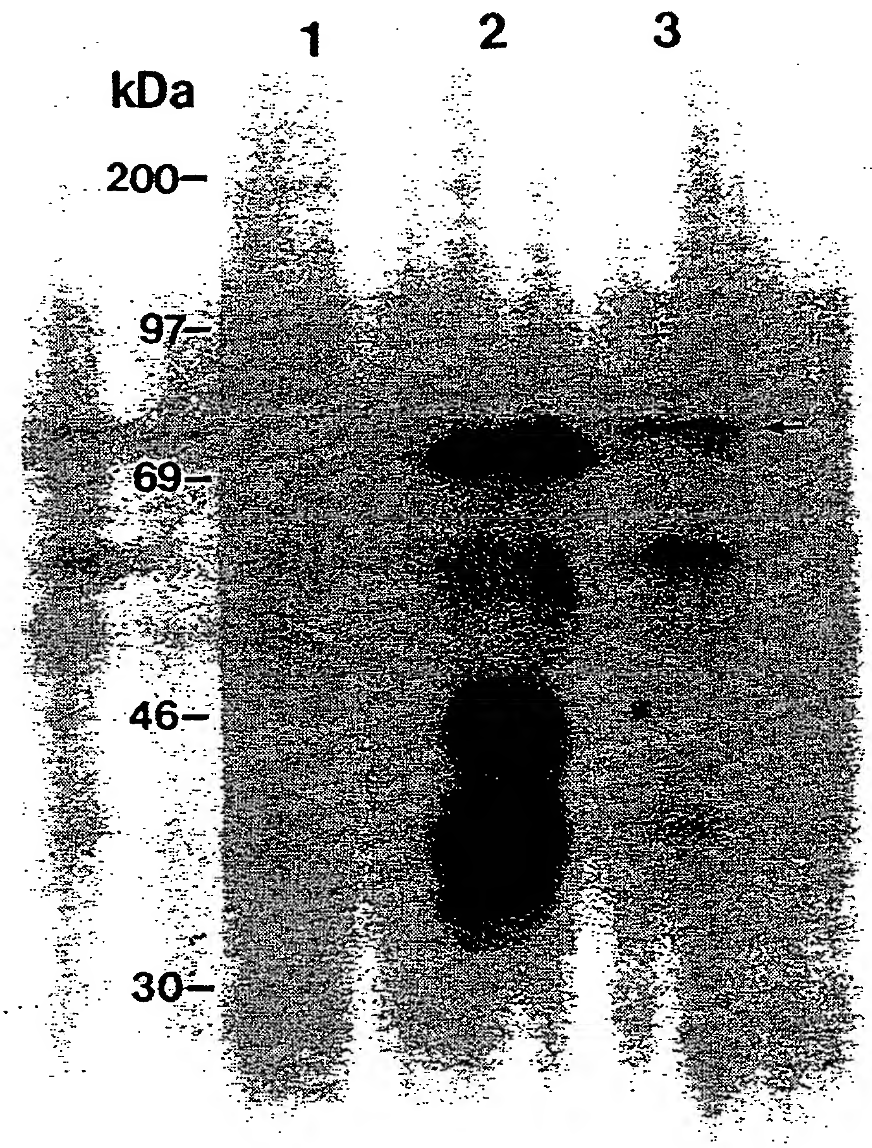


FIG 13B

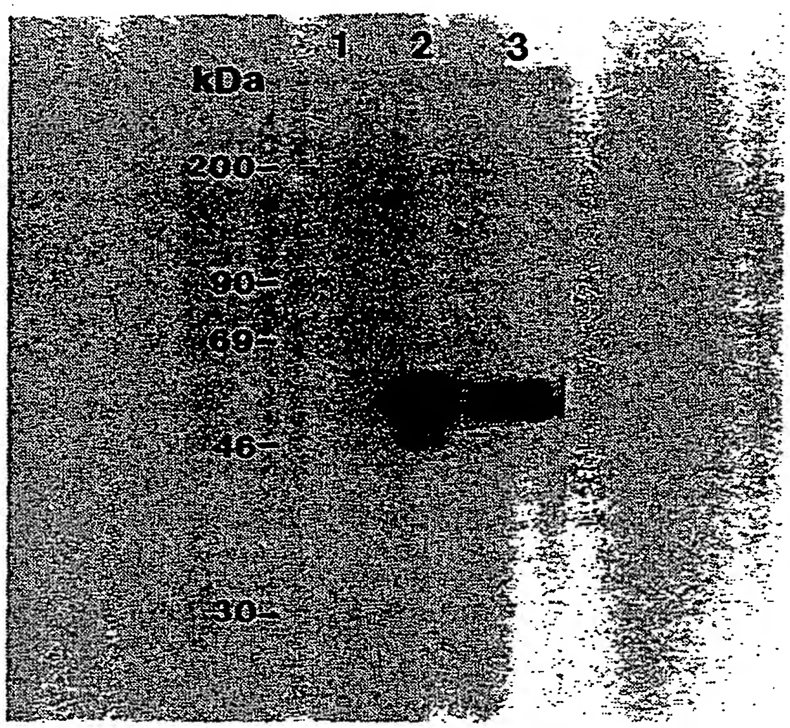


FIG 14A

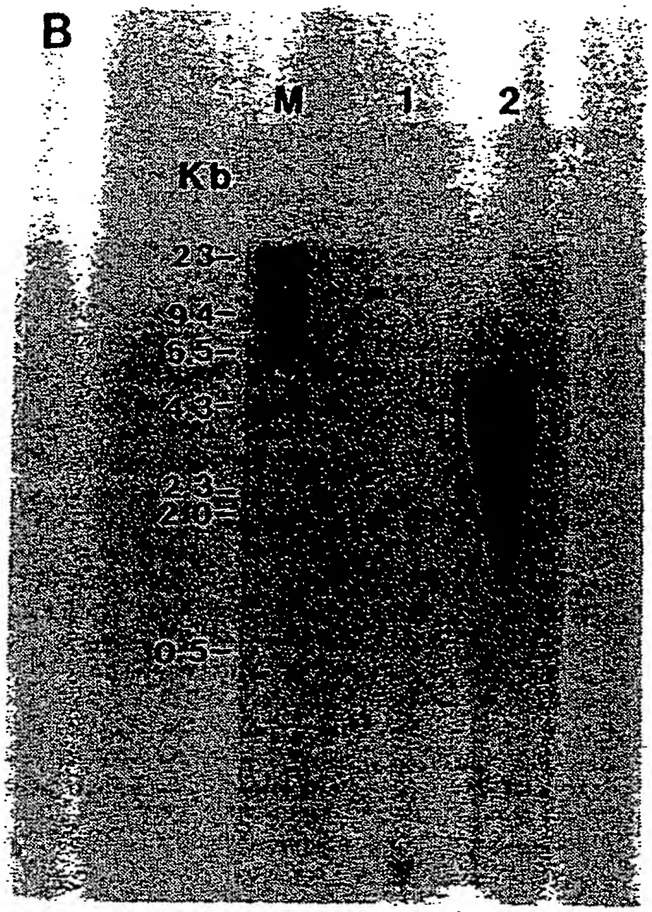


FIG 14B

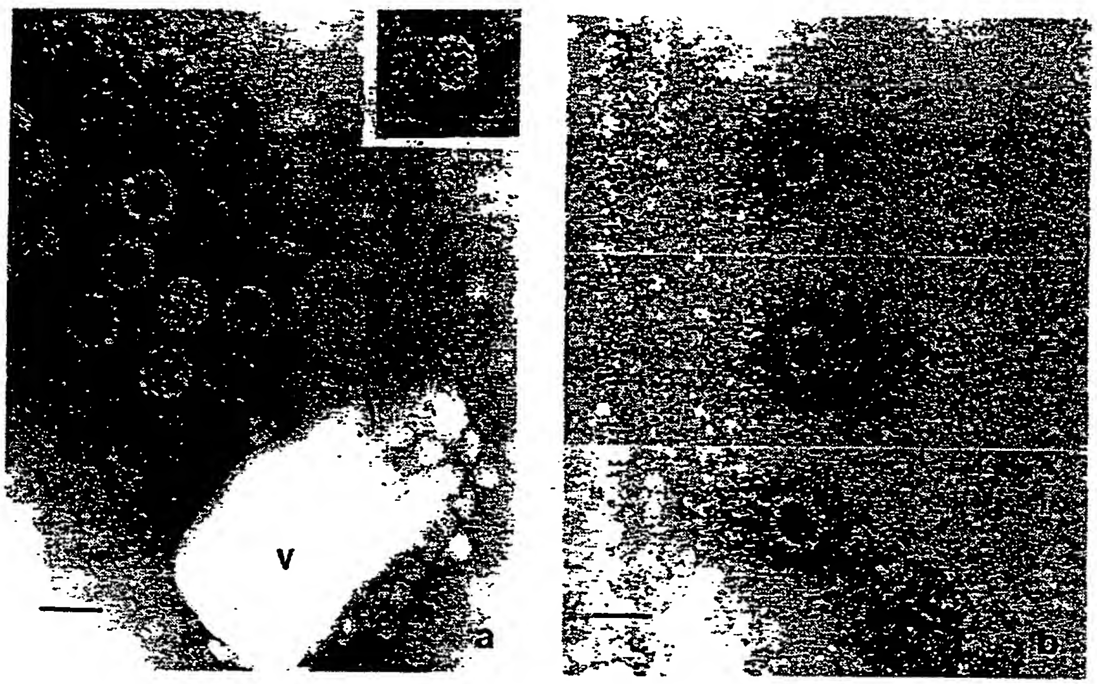


FIG 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00364 421

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.^{5} C12N 7/01, 7/04, 15/12, 15/37, A61K 39/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols) IPC:C12N, A61K
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU:C12N 7/01, 7/04, 15/37
Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) DERWENT DATABASE; WPAT - KEYWORDS; VIRUS OR VIRION - LIKE PARTICLES, PAPILLOMA, L1, L2, VLP CHEMICAL ABSTRACTS: CASA-KEYWORDS AS ABOVE: STN DATABASE: NUCLEOTIDE SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Chemical Abstracts, Volume 13, No 19, issued 1990, November 5 (Columbus, Ohio, USA.), Abstract No. 170012Z Zhou et al.,"Increased antibody responses to human papillomavirus type 16 L1 protein expressed by recombinant vaccinia virus lacking serine protease inhibitor genes." J. Gen. Virol 1990, 71(9), 2185-90	33
P,X	Virology, Volume 185, No 1, 1991, ZHOU J et al "Expression of vaccinia reccombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles" pp 251-257 whole article	1-38

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 1992 (29.10.92)	4 NOV. 1992 (04.11.92)
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer Ross Osborne R OSBORNE Telephone N . (06) 2832313

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim N .
A	WO, A, 91/04330 (RIJKSUNIVERSITEIT TE LEIDEN) 4 April 1991 (04.04.91)	